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14. ABSTRACT
We hypothesized that dehydroepiandrosterone metabolites or their synthetic derivatives are able to bind to the androgen receptor with low, if any, agonist activity and thus function as better antiandrogens than currently available ones. We preliminarily identified three potential dehydroepiandrosterone derivatives with marginal androgenic activity. In this project, we assessed the effects of these compounds, in comparison with classic antiandrogens clinically used, on cell proliferation/apoptosis, cell migration/invasion, and prostate-specific antigen expression in prostate cancer lines *in vitro* as well as on tumor growth in animal models for prostate cancer and found their inhibitory effects on androgen-mediated tumor outgrowth. We further dissected molecular mechanisms of how such compounds suppress the progression of prostate cancer and found that they could alter androgen-mediated androgen receptor functions in prostate cancer cells. Importantly, these dehydroepiandrosterone derivatives were found to possess marginal agonist effects on the activity of androgen receptor.

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1. Introduction

Although antiandrogens that can block androgen action through the androgen receptor (AR) have been widely used for the treatment of prostate cancer, the majority of available agents possess agonist activity, resulting in increases in serum prostate-specific antigen (PSA) levels, known as the antiandrogen withdrawal syndrome [1,2]. In addition, we previously demonstrated that androstenediol, a physiological metabolite from dehydroepiandrosterone (DHEA) and a precursor of testosterone, has an intrinsic androgenic activity which was not completely antagonized by two antiandrogens clinically used, flutamide and bicalutamide (BC) [3]. Therefore, new and more effective antiandrogenic compounds with marginal androgenic activities need to be identified. Our hypothesis in the current project was that DHEA metabolites or their synthetic derivatives could bind to the AR with low, if any, agonist activity and might thus function as better antiandrogens than currently available ones. We previously screened DHEA metabolites/derivatives for their androgenic and antiandrogenic activities and found that three compounds, 3β-acetoxyandrost-1,5-diene-17-ethylene-ketal (ADEK), 3β-hydroxyandrost-5,16-diene (HAD), and 3-oxo-androst-1,4-diene-17-ketal (OADK), showed only marginal agonist effects and suppressed significantly 5α-dihydrotestosterone (DHT)- and androstenediol-induced AR transcriptional activities [4-6]. Accordingly, ADEK, HAD, and OADK have the potential to function as potent antiandrogens that carry fewer risks of withdrawal response if used for therapy in patients with prostate cancer. In this project, we have assessed the effects of these DHEA derivatives, in comparison with classic antiandrogens clinically used, on cell proliferation/ apoptosis, cell migration/invasion, and prostate-specific antigen expression in prostate cancer lines *in vitro* as well as on tumor growth in animal models for prostate cancer. We have further dissected molecular mechanisms of how such compounds suppress tumor growth, presumably altering androgen-mediated AR functions in prostate cancer cells.

2. Keywords

3β-acetoxyandrost-1,5-diene-17-ethylene-ketal;
androgen receptor;
androgen receptor coregulator;
androstenediol;
antiandrogen withdrawal syndrome;
bicalutamide;
dehydroepiandrosterone;
dihydrotestosterone;
3β-hydroxyandrost-5,16-diene;
hydroxyflutamide;
mouse xenograft model;
3-oxo-androst-1,4-diene-17-ketal;
prostate cancer;
prostate-specific antigen; and
testosterone

3. Overall Project Summary

Effects of ADEK, HAD, and OADK on cell proliferation (Task 1a)

Using MTT (thiazolyl blue) assay, we first examined androgenic/antiandrogenic effects of ADEK, HAD, and OADK, in comparison with those of hydroxyflutamide (HF), on cell proliferation of six prostate cancer cell lines with different AR statuses. LNCaP and CWR22Rv1 express a mutant AR T877A and a mutant AR H874Y, respectively. PC-3(AR)2 and PC-3(AR)9 are stable clones of AR-negative PC-3 expressing wild-type AR under control of a cytomegalovirus promoter [7] and a natural AR promoter [6,8], respectively. These cell lines were cultured for 6 days in the presence or absence of 1 nM DHT and different concentrations of HF, ADEK, HAD, or OADK. In LNCaP, DHT or HF increased cell growth by nearly 100% after 6-day culture (Fig. 1A, lanes 1 *vs.* 2 or 9), whereas ADEK, HAD, and OADK, except 1 μM OADK, showed marginal (<10%) growth induction in the absence of androgens (lanes 1 *vs.* 3-8). ADEK (0.1 and 1 μM), HAD (1 μM), and OADK (0.1 μM), but not HF, significantly antagonized the effect of DHT (lanes 9 *vs.* 10-16). Similarly, in CWR22Rv1, HF, ADEK, HAD, and OADK, except 1 μM OADK, showed marginal (<10%) growth induction in the absence of androgens (Fig. 1B, lanes 1 *vs.* 2-8). HF (1 μM), ADEK (0.1 and 1 μM), HAD (1 μM), and OADK (0.1 μM) significantly antagonized the DHT effect (lanes 9 *vs.* 10-16). In PC-3(AR)9, DHT increased cell growth by only 13% (Fig. 1D, lanes 1 *vs.* 9). Although HF, ADEK, HAD, and OADK showed marginal (<7%) growth induction in the absence of androgens (lanes 1 *vs.* 2-8), these compounds did not significantly antagonize the effect of DHT (<6%; lanes 9 *vs.* 10-16). In PC-3(AR)2 (Fig. 1C), PC-3 (Fig. 1E), and DU145 (Fig. 1F), DHT, HF, and/or each of the 3 steroid derivatives showed marginal effects on cell growth. We also performed MTT assay in the same cell lines with treatment of androstenediol (instead of DHT) and the antiandrogenic compounds. However, up to 10 nM of androstenediol (physiological concentrations in men are ~5 nM [3]) did not significantly increase the growth of any of the six cell lines and, therefore, only marginal suppression by steroid derivatives were seen (figure not shown).
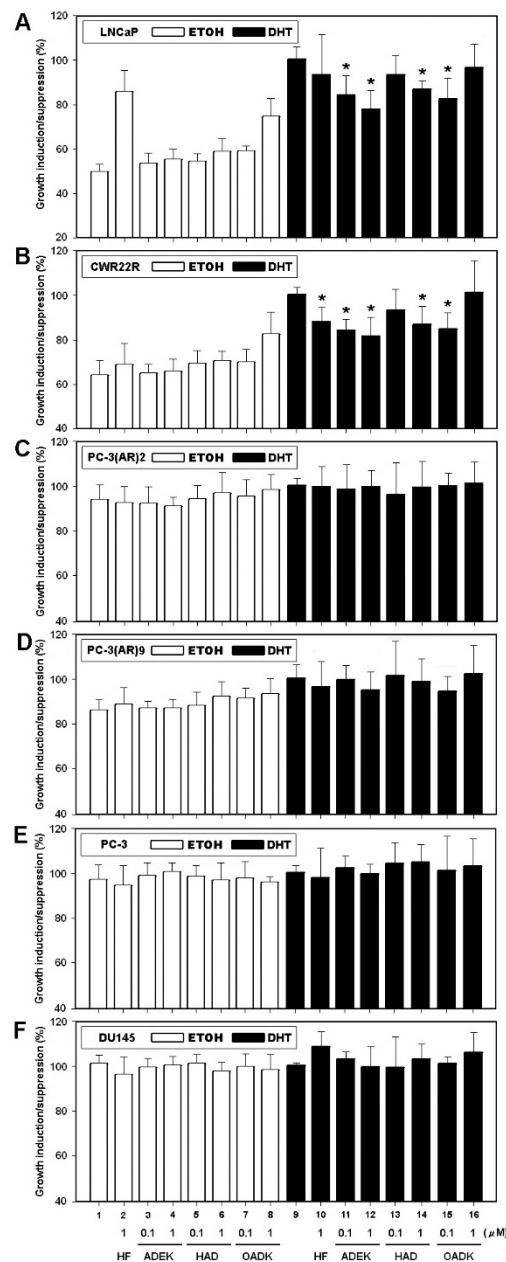
Figure 1. The effects of DHEA derivatives on cell proliferation. LNCaP (A), CWR22Rv1 (B), PC-3(AR)2 (C), PC-3(AR)9 (D), PC-3 (E), or DU145 (F) cells were cultured for 6 days with different concentrations of HF, ADEK, HAD, OADK in the absence [ethanol (ETOH); white bars] or presence (black bars) of 1 nM DHT, as indicated. The MTT assay was performed and growth induction/suppression is presented relative to cell number with DHT treatment in each panel (ninth lanes; set as 100%). Values represent the mean + SD of at least three determinations. *p<0.05 (*vs.* DHT for lanes 10-16; analyzed by Student's *t*-test).

Anti-DHT effects of ADEK, HAD, and OADK on apoptosis (Task 1a)
Using DNA fragmentation (TUNEL) assay, we next assessed antiandrogenic effects of ADEK, HAD, and OADK on apoptosis. Prostate cancer cell lines were cultured for 6 days with 1 nM DHT and different concentrations of HF (1 μM only), ADEK, HAD, or OADK. Apoptotic indices were determined by fluorescence microscopy. As summarized in Table 1, ADEK (0.1, 1 μM), HAD (1 μM), and OADK (0.1, 1 μM), in the presence of DHT (1 nM), were found to induce apoptosis in LNCaP and CWR22Rv1 cells. In contrast, in PC-3 as well as PC-3(AR)2 and PC-3(AR)9 cells, there are no significant differences in apoptotic indexes between DHT with and without each antiandrogenic compound.

Table 1. Apoptosis in prostate cancer cell lines following the treatment of DHEA derivatives.

Treatment (+ 1 nM DHT)	LNCaP	CWR22R	PC-3(AR)2	PC-3(AR)9	PC-3
Mock (Ethanol)	1.6	2.7	5.0	3.9	1.8
HF (1 μM)	2.4	9.2	5.5	5.1	2.2
ADEK (0.1 μM)	12.8	16.4	5.8	6.9	1.9
ADEK (1 μM)	22.3	20.6	6.3	7.7	1.9
HAD (0.1 μM)	4.9	5.4	5.8	6.0	1.8
HAD (1 μM)	18.8	16.6	5.7	7.0	2.2
OADK (0.1 μM)	19.3	20.4	6.9	9.5	2.7
OADK (1 μM)	17.8	18.5	6.2	9.9	1.9

Apoptotic index = percentage of TUNEL-positive cells in a total of 1,000 cells.

Anti-DHT effects of ADEK, HAD, and OADK on colony formation (Task 1a)
We determined the cell growth by a colony formation assay. The number of colonies formed in LNCaP (Fig. 2A) or CWR22Rv1 (Fig. 2B) cells was significantly augmented by DHT treatment. However, the DHEA derivatives as well as HF showed marginal to only slight reductions in LNCaP (up to 13%) and CWR22Rv1 (up to 12%). These results suggest that, inconsistent with the cell viability data (Fig. 1), antiandrogens tested do not significantly inhibit androgen-induced colony formation of prostate cancer cells.
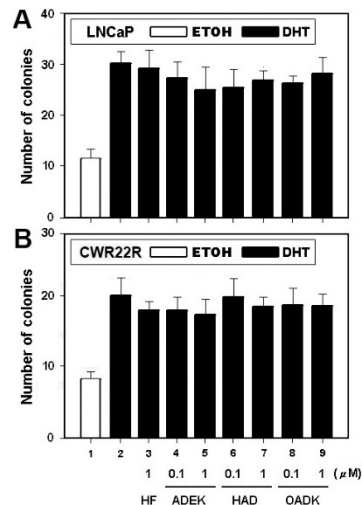
Figure 2. The effects of DHEA derivatives on colony-forming. LNCaP (A) or CWR22Rv1 (B) cells plated onto the soft agar were cultured for 2 weeks with different concentrations of HF, ADEK, HAD, OADK in the absence [ethanol (ETOH); white bar] or presence (black bars) of 1 nM DHT, as indicated, and stained with methylene blue. Colonies with cell numbers higher than 50 were counted. Values represent the mean + SD of at least three determinations.

Anti-DHT effects of ADEK, HAD, and OADK on cell migration/invasion (Task 1a)
We determined the invasion ability of prostate cancer cells using the transwell chamber assay. As shown in Figs. 3A and 3B, DHT enhanced the invasion ability in LNCaP (40% increase) and CWR22Rv1 (59% increase) cells. However, the DHEA derivatives and HF showed marginal to

only slight reductions in the presence of DHT. In PC-3(AR)2 (figure not shown), PC-3(AR)9 (Fig. 3C), or PC-3 (figure not shown), DHT marginally (up to 16%) increased the invasion, and DHEA derivatives did not show significant inhibitory effects. These results suggest that antiandrogens tested do not significantly inhibit androgen-induced cell migration/invasion of prostate cancer cells.

Figure 3. The effects of DHEA derivatives on invasive ability. Matrigel in serum-free cold cell culture medium was placed in the upper chamber of a 24-well transwell and incubated for 5 h at 37°C. LNCaP (A), CWR22Rv1, or PC-3(AR)9 (C) cells were harvested, and cell suspensions (100 µl) were placed on the matrigel, and the lower chamber of the transwell was filled with culture medium in the presence of 5 µg/ml fibronectin, as an adhesive substrate. DHT (1 nM) together with different concentrations of HF, ADEK, HAD, or OADK was added in both upper and lower chambers. Following 48 h of incubation at 37°C, and invading cells were stained with Giemsa solution and counted under the microscope. Values represent the mean + SD of at least three determinations.
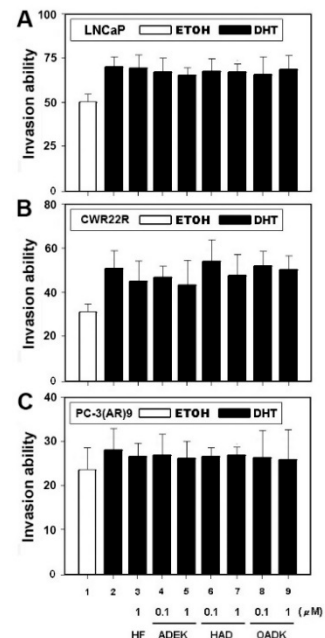
Anti-DHT effects of ADEK, HAD, and OADK on mRNA expression of PSA, AR, and other genes related to angiogenesis/metastasis (Task 1a)

A quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis was performed in AR-positive/PSA-positive prostate cancer cells, LNCaP and CWR22Rv1, in order to assess the antiandrogenic effects of ADEK, HAD, and OADK on tumor progression (*i.e.* PSA, AR) and angiogenesis/metastasis [*i.e.* basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin (IL)-6, and matrix metalloproteinase (MMP)-9]. As expected, DHT increased PSA expression, which was suppressed by ADEK (1 µM), HAD (1 µM), and OADK (0.1 µM) in both LNCaP and CWR22Rv1 cells (Fig. 4A). DHT increased AR mRNA, and only ADEK (in LNCaP) or HAD (in CWR22Rv1) significantly inhibited the DHT-mediated AR expression, although all 3 compounds, as well as HF, showed a tendency to decrease it (Fig. 4B). Similarly, DHT could significantly increase the expression of bFGF (Fig. 4C), VEGF (Fig. 4D), IL-6 (Fig. 4E), and MMP-9 (Fig. 4F) in LNCaP and CWR22Rv1 cells. However, some treatments significantly antagonized the effects of DHT (ADEK/ HAD for VEGF in LNCaP, HAD for VEGF in CWR22Rv1, and ADEK for IL-6 or MMP-9 in CWR22Rv1). Again, 10 nM androstenediol did not significantly increase the expression of these genes, which was only marginally suppressed by the steroid derivatives (figure not shown).
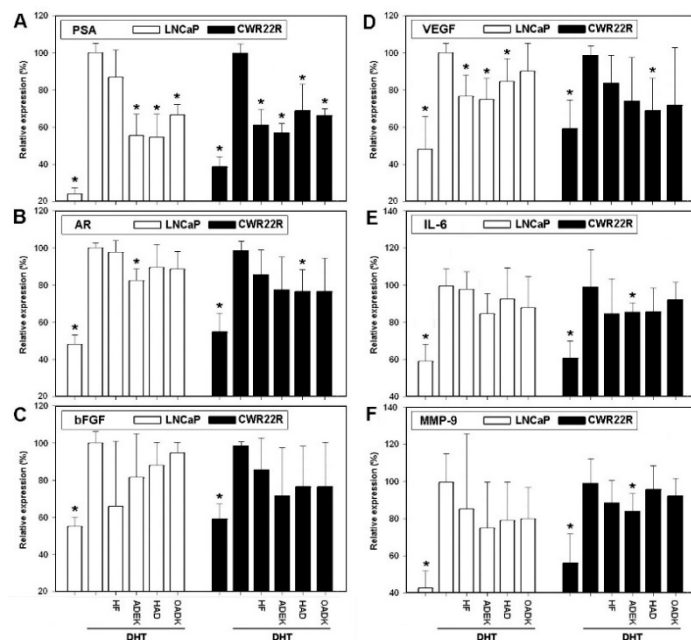
Figure 4. The effects of DHEA derivatives on the expression of PSA (A), AR (B), bFGF (C), VEGF (D), IL-6 (E), and MMP-9 (F). LNCaP (white bars) or CWR22Rv1 (black bars) cells were cultured for 48 h with 1 µM HF, 1 µM ADEK, 1 µM HAD, or 0.1 µM OADK in the absence or presence of 1 nM DHT, as indicated. Total RNAs from these cells were isolated and reverse transcribed. Real-time PCR was then performed, using each specific primer set. GAPDH was used as an internal control. Expression levels are presented relative to those with DHT treatment in each panel (second lanes; set as 100%). Values represent the mean + SD of at least three determinations. *p<0.05 (*vs.* DHT; analyzed by Student's *t*-test).

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Anti-DHT effects of ADEK, HAD, and OADK on protein expression of PSA (Task 1a)

Western blotting analysis was performed to determine whether the DHEA derivatives inhibit androgen-mediated PSA protein expression in prostate cancer cells. As expected, DHT increased endogenous PSA expression in LNCaP or CWR22Rv1 cells over mock treatment (Fig. 5, lanes 1 *vs.* 2). ADEK, HAD, and OADK showed only marginal induction without androgens (lanes 1 *vs.* 3, 5, or 7) and antagonized DHT-induced PSA expression (lanes 2 *vs.* 4, 6, or 8). HF did not show significant anti-DHT effects (lanes 2 *vs.* 10).
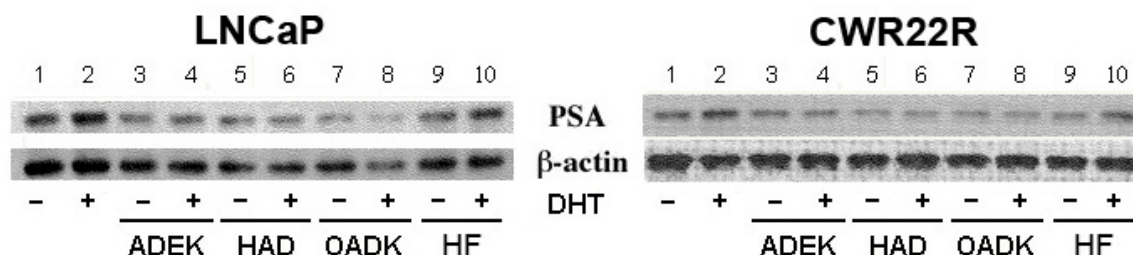

Figure 5. The effects of DHEA derivatives on PSA expression. Cell extracts from LNCaP or CWR22Rv1 cultured for 48 h with ADEK (1 μM), HAD (1 μM), OADK (0.1 μM), or HF (1 μM) in the absence or presence of 1 nM DHT, as indicated, were analyzed on Western bots, using an antibody to PSA (upper) or β-actin (lower). The 33 kDa (for PSA) and 43 kDa (for β-actin as an internal control) proteins were detected.

Anti-DHT effects of ADEK, HAD, and OADK on protein expression of AR and other molecules related to angiogenesis and metastasis (Task 1a)

Additional Western blotting analyses were performed to assess whether the DHEA derivatives inhibit androgen-induced protein expression of AR and angiogenic/metastatic factors, including VEGF, IL-6, and MMP-9, in prostate cancer cells. As expected, DHT increased endogenous expression of AR and other factors in LNCaP or CWR22Rv1 cells over mock treatment (Fig. 6). Then, ADEK, HAD, and OADK showed antiandrogenic effects on the expression of most of the proteins without significant agonist activities.

Figure 6. The effects of DHEA derivatives on the expression of proteins related to angiogenesis/ metastasis. Cell extracts from LNCaP or CWR22Rv1 cultured for 48 h with ADEK (1 μM), HAD (1 μM), or OADK (0.1 μM) in the absence or presence of 1 nM DHT, as indicated, were analyzed on Western bots, using an antibody to AR, VEGF, IL-6, MMP-9, or β-actin.
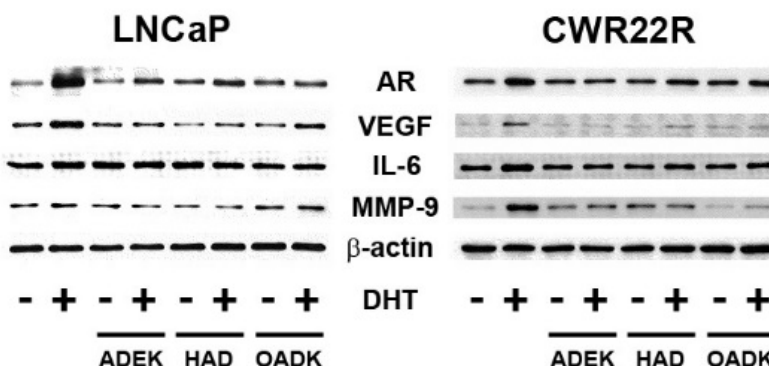
Effects of long-term treatment with ADEK, HAD, or OADK (Task 1b)

To see if long-term culture with each DHEA derivative leads to any changes in the cells (*e.g.* growth rate, AR or PSA expression, response to androgen supplementation), we have cultured LNCaP cells for at least 20 weeks with 1 μM ADEK, 1 μM HAD, or 1 μM OADK. Using these sublines, we then performed MTT assay, RT-PCR, and Western blotting, as described above. However, we identified no significant differences in cell growth in the presence or absence of androgen and the expression of androgen-regulated proteins between each subline versus control subline.

Tolerance/toxicity for ADEK, HAD, and OADK in animals (Task 2a)
To determine whether the DHEA derivatives are well tolerated or affected any adverse responses in animals, ADEK, HAD, or OADK (200 mg/Kg daily for 14 days; 2x postulated therapeutic dose [9]) was administered subcutaneously in 6-week-old male C57BL/6 mice. The data in Table 2 demonstrate that there were no statistically significant differences (by Student's *t*-test) in food intake, weight gain, and weights of the heart, liver, kidney, adrenal, spleen, testis, and brain between control and treatment groups. These organs were also histologically examined, but there were no significant morphological changes in the H&E stained tissues from different groups of mice. Thus, it was likely that animals with injections of a high dose of ADEK, HAD, or OADK suffered from no adverse effects.

Table 2. Responses to injected DHEA derivatives in mice.

|	Control	ADEK	HAD	OADK
Weight gain (g)	1.7	1.5	1.6	1.6
Food intake (g)	55.8	52.1	52.7	55.0
Heart (mg)	120	109	111	118
Liver (mg)	1,180	1,221	1,215	1,264
Kidney (R+L, mg)	387	402	398	380
Adrenal (R+L, mg)	12	11	10	11
Spleen (mg)	78	74	77	81
Testis (R+L, mg)	365	339	342	351
Brain (mg)	416	423	424	410

The data represent the average weights from 5 mice.

Anti-tumor effects of ADEK, HAD, and OADK in mouse xenograft models for prostate cancer (Tasks 2b and 2c)
Inhibitory effects of the DHEA derivatives on tumor growth have been assessed in mouse xenograft models for AR-positive prostate cancer. We used LNCaP and CWR22Rv1 because the compounds were found to significantly suppress androgen-mediated cell proliferation *in vitro*. These lines were implanted subcutaneously into the flanks of 7-8-week-old male SCID mice. After 2-4 weeks when the estimated volumes of all tumors reached 40 mm^3, we started daily injection of ADEK, HAD, or OADK into mice. Because daily injections of each compound at 100 mg/Kg resulted in marginal decreases in tumor size (data not shown; detailed in the Annual Report of July 2012), we used a higher dose (200 mg/Kg) that remained tolerable in animals (see above). As shown in Fig. 7, inoculated LNCaP/CWR22Rv1 tumors in mice treated with BC (without castration) were significantly smaller (79%/51% reductions at 10/8 weeks, respectively) than those in the control mice. Treatment of ADEK (39%/29% at 10/8 weeks), HAD (29%/20% at 10/8 weeks), or OADK (29%/24% at 10/8 weeks) reduced the size of the LNCaP/CWR22Rv1 tumors, compared with control treatment, but the differences were not statistically significant (*P*>0.05). In

addition, castration (bilateral orchiectomy) significantly retarded the growth of the LNCaP tumors, and no significant additive effects of BC or each DHEA derivative were seen.

Figure 7. The effects of ADEK, HAD, and OADK on tumor progression in mouse xenograft models for prostate cancer. LNCaP (**A**) or CWR22Rv1 (**B**) cells resuspended in Matrigel (2 × 10⁶ cells in 200 μl per site) were implanted
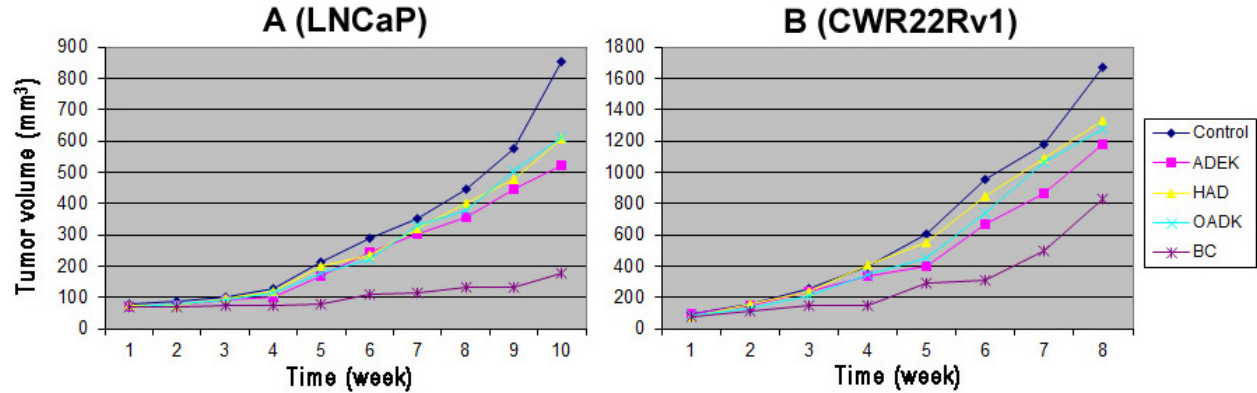
subcutaneously into the flanks of SCID mice treated with daily intra-peritoneal injection of 200 mg/Kg each compound. Tumor volume (n = 6 tumors in each group) calculated by the following formula: tumor weight = tumor length (mm) × [tumor width (mm)]² × 0.5 [10] was then monitored twice a week for 8-10 weeks.

Some of the harvested tumor specimens were then assessed for cell proliferation [by proliferating cell nuclear antigen (PCNA) immunostaining; Fig. 8], apoptosis (by TUNEL assay; Fig. 8), and angiogenesis or metastatic ability [micro-vessel density (MVD) by CD31 immunostaining; Fig. 8) as well as the expression of bFGF, VEGF, IL-8, and MMP-9 by quantitative RT-PCR; Fig. 9]. Correlating with the sizes of xenograft tumors, BC or castration, but not ADEK, HAD, or OADK, significantly changed these parameters. There were also no noticeable differences in the expression of VEGF, MMP-9, and E-cadherin detected by immunohistochemistry, as well as that of VEGF, MMP-2, and MMP-9 detected by Western blotting, between the tumors from the control versus ADEK/HAD/OADK groups (figure not shown).
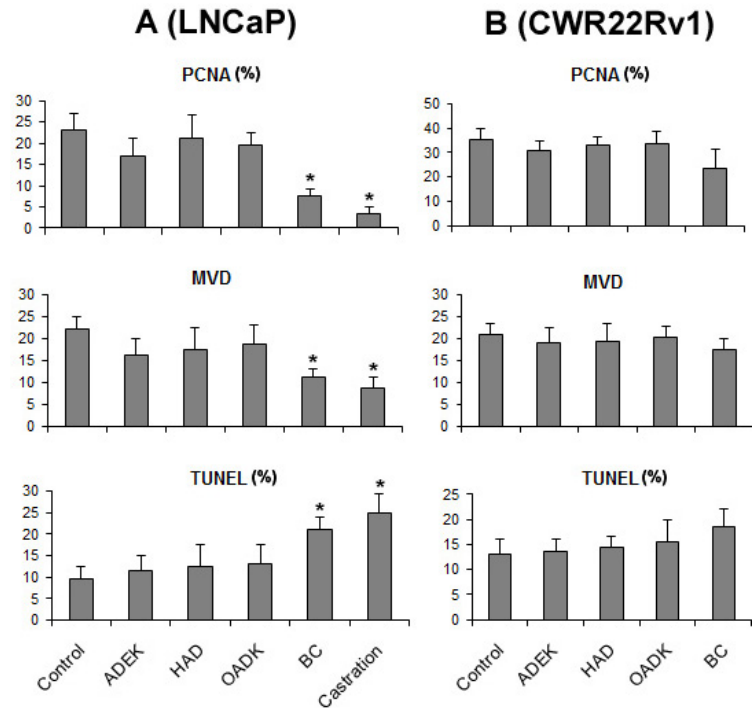
Figure 8. The effects of ADEK, HAD, and OADK on cell proliferation, angiogenesis, and apoptosis in mouse xenograft models for prostate cancer. The LNCaP (**A**) and CWR22Rv1 (**B**) xenograft tumors described in Figure 7 were harvested for immunohistochemical and TUNEL analyses. Mean values + SDs of the percentage of PCNA-positive cells, MVD (number of vessels highlighted by CD31 staining per high-power field), and the percentage of TUNEL-positive cells in each group of tumors are shown. *P<0.05 (vs. control by Student's t-test).

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Figure 9. The effects of ADEK, HAD, and OADK on angiogenesis- and/or cell invasion-related genes in mouse xenograft models for prostate cancer. The LNCaP (**A**) and CWR22Rv1 (**B**) xenograft tumors described in Figure 7 were harvested for RNA extraction. The expression of bFGF, VEGF, IL-8, and MMP-9 mRNAs in the tumors was analyzed by real-time RT-PCR. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that of control tumors in each cell line (first lanes; set as 100%). Values represent the mean + SD from at least three independent experiments. *$P<0.05$ (*vs.* control by Student's *t*-test).
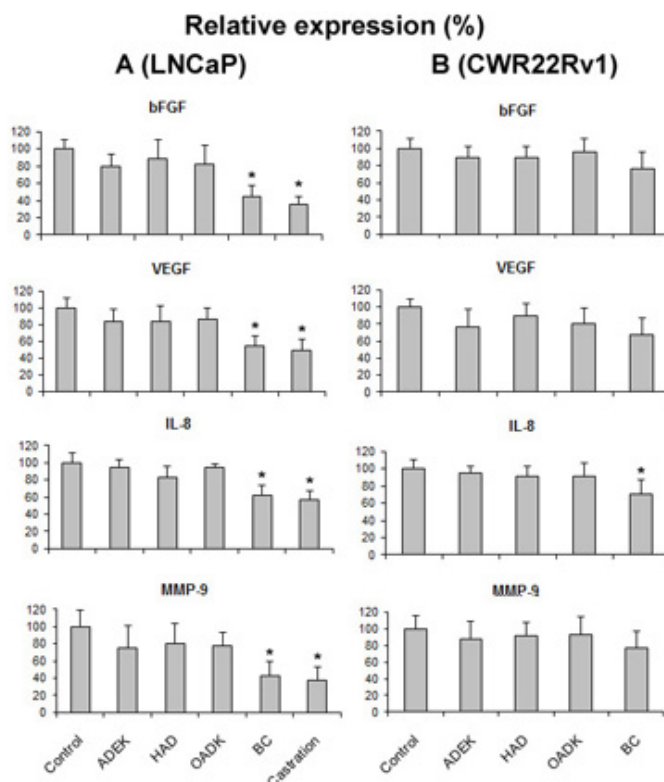
Thus, in contrast to our *in vitro* data, ADEK, HAD, and OADK did not show significant suppressive effects on AR-positive tumor growth *in vivo*. We repeated mouse xenograft experiments, using another prostate cancer cell line, VCaP, harboring a wild-type AR. However, the tumors grew much more slowly in mice, and inhibitory effects of the DHEA derivatives as well as BC on tumor growth were not apparent (figure not shown).

Anti-carcinogenic effects of ADEK, HAD, and OADK in the TRAMP model

Alternatively, as proposed in Specific Aim 2 (Alternative approach 3), chemopreventive effects of the DHEA derivatives were assessed in the TRAMP transgenic mouse model in which a premalignant lesion [prostatic intraepithelial neoplasia (PIN)], invasive prostatic adenocarcinoma, and metastasis are sequentially developed [11]. At the age of 5 weeks prior to tumor development in the prostate, each compound at 100 mg/Kg was injected daily into the mice (n=8/group at each time point). The mice were then sacrificed at 12 and 24 weeks, and the prostates were histologically assessed. As summarized in Table 3, ADEK, OADK, or HAD did not significantly prevent the development of PIN and invasive cancer. At 24 weeks ADEK reduced the incidence of prostate cancer from 88% to 50% (*P*=0.282).

Table 3. The incidence of prostatic adenocarcinoma in the TRAMP mice.

Group	PIN at 12 weeks	Cancer at 12 weeks	Cancer at 24 weeks
Control	7 (88%)	3 (38%)	7 (88%)
ADEK	5 (63%)	2 (25%)	4 (50%)
HAD	6 (75%)	3 (25%)	6 (75%)
OADK	7 (88%)	3 (38%)	5 (63%)

Binding affinity of ADEK, HAD, and OADK for the AR (Task 3a)

To determine whether the DHEA derivatives have an affinity for the AR, allowing a competition with androgens for binding, competitive androgen binding assay was performed in LNCaP with endogenous mutant AR and DU145 with transfected wild-type AR. As described [5,6], the relative binding affinity (RBA) values were calculated. Competitive RBAs in both LNCaP and DU145

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with AR were: DHT > BC > OADK > ADEK > HAD (Table 4). These results confirm that the DHEA derivatives, particularly ADEK and OADK, are able to compete significantly with androgens for AR binding.

Table 4. AR ligand binding affinity.

| Ligand	RBA in LNCaP	RBA in DU145 with AR
DHT	100.0	100.0
BC	48.1	28.8
ADEK	10.5	5.5
HAD	2.1	1.2
OADK	14.5	12.4

Effects of ADEK, HAD, and OADK on AR protein expression and its stability (Tasks 3c and 3d)

We showed that ADEK, HAD, and OADK inhibited androgen-induced expression of AR in LNCaP and CWR22Rv1 cells while they did not reduce AR expression in the absence of androgens [Fig. 4 & Ref. 12]. To further determine whether the DHEA derivatives affect the stability of AR mRNA and protein, quantitative RT-PCR and Western blotting analyses were performed in LNCaP and CWR22Rv1 cell lines pretreated with actinomycin D or cycloheximide. In these experiments, however, there were no significant differences in the ratios of AR expression/ degradation between the control versus ADEK/HAD/OADK groups in the presence and absence of DHT (figure not shown). These findings suggest that the DHEA derivatives have little influence on AR stability in prostate cancer cells.

Effects of ADEK, HAD, and OADK on AR NH$_2$-/COOH-terminal (N/C) interaction (Task 3e)

It is well documented that AR N/C interaction is important for full AR activation [13]. We therefore assessed whether the DHEA derivatives exert an influence on the interaction, using mammalian two-hybrid assay, in AR-negative prostate cancer cells. PC-3 and DU145 cells were transfected with a GAL4-hybrid plasmid expressing AR-DNA binding domain/ligand binding domain, a VP16-hybrid plasmid expressing AR-NH$_2$-terminus, and a luciferase reporter plasmid (pG5-Luc), and treated with DHT and each antiandrogenic compound (Fig. 10). As expected, DHT induced AR N/C interactions in both cell lines. DHEA derivatives only marginally increased the luciferase activity (except HAD in PC-3; 4.5-fold over mock treatment, $P<0.05$) and significantly reduced DHT-enhanced activities. Thus, it was likely, as seen in BC, that ADEK, HAD, and OADK inhibited androgen-mediated AR N/C interactions in prostate cancer cells.

Figure 10. The impact of DHEA derivatives on AR N/C interaction. PC-3 or DU145 cells were transfected with pCMX-GAL4-AR-C, VP16-AR-N, pG5-Luc, and pRL-TK, and subsequently cultured in the presence or absence of 1 nM DHT, 10 µM BC, 1 µM ADEK, 1 µM HAD, and/or 1 µM OADK, as indicated. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit and luminometer. Luciferase activity is presented relative to that of mock treatment in each cell line. Values represent the mean + SD from four independent experiments.
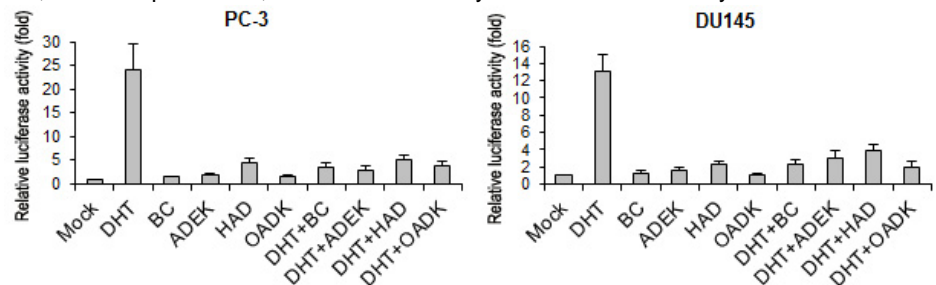
Effects of ADEK, HAD, and OADK on AR-AR coregulator interactions (Task 3f)

It has also been known that maximal or proper androgen action via AR requires the interactions between AR and selective AR coregulators [14]. We therefore assessed whether the DHEA derivatives exert an influence on the interactions, using mammalian two-hybrid assay, in prostate cancer cells. DU145 cells were transfected with a GAL4-hybrid plasmid expressing AR-DNA binding domain/ligand binding domain, a VP16-hybrid plasmid expressing each AR coregulator, and a luciferase reporter plasmid (pG5-Luc), and treated with DHT as well as each antiandrogenic compound (Fig. 11). As expected, DHT induced AR interaction with each AR coregulator. As reported [14-16], BC also promoted the interactions between AR and ARA70 or ARA54 (but not between AR and ARA55 or SRC-1 in our assays). Similarly, DHEA derivatives significantly (*P*<0.05) induced some of the interactions (ADEK: ARA70 and ARA54; HAD: ARA70, ARA54, and ARA55; and OADK: ARA70 and ARA54). Nonetheless, these compounds inhibited all of the DHT-induced interactions. Thus, it was likely, as seen in BC, that ADEK, HAD, and OADK inhibited androgen-mediated interactions between AR and AR coregulators in prostate cancer cells, while they also had some agonist effects.
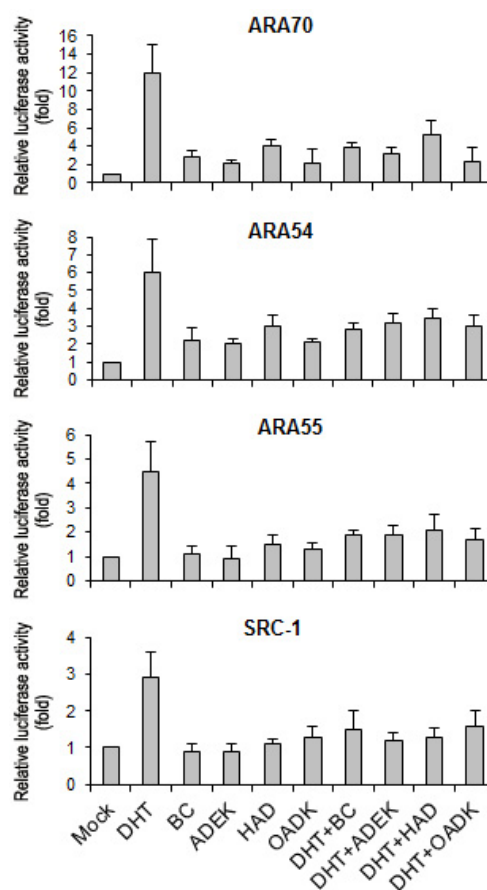
Figure 11. The impact of DHEA derivatives on AR-AR coregulator interactions. DU145 cells were transfected with pCMX-GAL4-AR-C, VP16-ARA70/ARA54/ARA55/SRC-1, pG5-Luc, and pRL-TK, and subsequently cultured in the presence or absence of 1 nM DHT, 10 µM BC, 1 µM ADEK, 1 µM HAD, and/or 1 µM OADK, as indicated. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit and luminometer. Luciferase activity is presented relative to that of mock treatment. Values represent the mean + SD from at least three independent experiments.

Effects of ADEK, HAD, and OADK on phosphorylation status of AR (Task 3g)

The AR exists as a phosphoprotein and modulation of the phosphorylation status of the receptor affects ligand-binding and subsequent transcriptional activation of androgen responsive genes [17,18]. We therefore assessed whether the DHEA derivatives inhibit AR transactivation via alteration of AR phosphorylation. Western blotting was performed in LNCaP cells treated with DHT as well as ADEK, HAD, or OADK, using a phospho-specific AR antibody. However, ADEK, HAD, and OADK appeared to only marginally change the phosphorylation status of AR in prostate cancer cells (figure not shown).

Effects of ADEK, HAD, and OADK on nuclear translocation of AR (Task 3h)

Immunofluorescent staining was performed to assess the effects of DHEA derivatives on nuclear translocation of AR in prostate cancer cells. PC-3 cells cultured in the presence or absence of DHT, ADEK, HAD, and/or OADK were subjected to immunofluorescence with an anti-AR antibody. As described in BC [19], DHEA derivatives did not strongly block the receptor nuclear translocation induced by DHT (figure not shown). These findings were confirmed by subcellular

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fractionation of nuclear and cytoplasmic proteins followed by Western blotting (figure not shown).

4. Key Research Accomplishments

- ADEK, HAD, and OADK were found to antagonize the effects of androgen on the proliferation, PSA expression, and AR transcriptional activity, without showing agonist activities, in prostate cancer cells.

- ADEK, HAD, and OADK were found to inhibit interactions between AR N- and C- terminuses as well as AR and AR coregulators in prostate cancer cells.

5. Conclusion

Using preclinical models for prostate cancer, we have demonstrated our data indicating that some DHEA derivatives, including ADEK, HAD, and OADK, function as AR antagonists and thereby inhibit androgen-mediated tumor growth. However, in some assays, these compounds have been found to be not superior to antiandrogens currently used in patients with prostate cancer. Importantly, ADEK, HAD, and OADK are found to possess only marginal androgenic activities. We have further shown that these compounds alter some of androgen-mediated AR functions in prostate cancer cells. These findings provide a basis for the development of novel, safe, and effective drugs for the treatment of advanced prostate cancer. However, further analyses of the compounds we have used as well as new DHEA derivatives in preclinical models are necessary prior to proceeding to clinical application.

6. Publications, Abstracts, and Presentations

(A) Lay Press:

Nothing to report.

(B) Peer-Reviewed Scientific Journals (the following articles acknowledge the current award):

1. Kawahara T, **Miyamoto H**: Androgen receptor antagonists in the treatment of prostate cancer. *Clin Immunol Endocr Metab Drugs* 1(1): 11-19, 2014.

2. Canacci AM, Izumi K, Zheng Y, Gordetsky J, Yao JL, **Miyamoto H**: Expression of semenogelins I and II and its prognostic significance in human prostate cancer. *Prostate* 71(10): 1108-1114, 2011. PMID: 21557275

3. Izumi K, Zheng Y, **Miyamoto H**: Eppin expression in prostate cancer. *Eur Urol* 59(6): 1071-1072, 2011. PMID: 21392882

4. Zheng Y, Izumi K, Yao JL, **Miyamoto H**: Dihydrotestosterone upregulates the expression of

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epidermal growth factor receptor and ERBB2 in androgen receptor-positive bladder cancer cells. *Endocr-Relat Cancer* 18(4): 451-464, 2011. PMID: 21613411

5. Li Y, Izumi K, **Miyamoto H**: The role of the androgen receptor in the development and progression of bladder cancer. *Jpn J Clin Oncol* 42(7): 569-577, 2012. PMID: 22593639

6. Izumi K, Li Y, Zheng Y, Gordetsky J, Yao JL, **Miyamoto H**: Seminal plasma proteins in prostatic carcinoma: increased nuclear semenogelin I expression is a predictor of biochemical recurrence after radical prostatectomy. *Hum Pathol* 43(11): 1991-2000, 2012. PMID: 22617231

7. Izumi K, Zheng Y, Li Y, Zaengle J, **Miyamoto H**: Epidermal growth factor induces bladder cancer cell proliferation through activation of the androgen receptor. *Int J Oncol* 41(5): 1587-1592, 2012. PMID: 22922989

8. Zheng Y, Izumi K, Li Y, Ishiguro H, **Miyamoto H**: Contrary regulation of bladder cancer cell proliferation and invasion by dexamethasone-mediated glucocorticoid receptor signals. *Mol Cancer Ther* 11(12): 2621-2632, 2012. PMID: 23033490

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10. Li Y, Zheng Y, Izumi K, Ishiguro H, Ye B, Li F, **Miyamoto H**: Androgen activates β-catenin signaling in bladder cancer cells. *Endocr-Relat Cancer* 20(3): 293-304, 2013. PMID: 23447569

11. Izumi K, Li Y, Ishiguro H, Zheng Y, Yao JL, Netto GJ, **Miyamoto H**: Expression of UDP-glucuronosyltransferase 1A in bladder cancer: association with prognosis and regulation by estrogen. *Mol Carcinogen* 54(4): 314-324, 2014. PMID: 23143693

12. Kawahara T, Kashiwagi E, Ide H, Li Y, Zheng Y, Ishiguro H, **Miyamoto H**: The role of NFATc1 in prostate cancer progression: Cyclosporine A and tacrolimus inhibit cell proliferation, migration, and invasion. *Prostate* 75(6): 573-584, 2015. PMID: 25631176

(C) Invited Articles:

Nothing to report.

(D) Abstracts:

1. Miyamoto H, Lai K-P, Zheng Y, Izumi K, Chang C: Identification of steroid derivatives as potent androgen receptor antagonists with marginal androgenic and estrogenic activity. Presented in IMPaCT2011 at Orlando, Florida (3/10/2011).

1. Reduced glucocorticoid receptor expression predicts bladder tumor recurrence and progression. 103rd Annual Meeting United States & Canadian Academy of Pathology, March 2014, San Diego, California; *Mod Pathol* 27(Suppl 2): 237A, 2014.

2. NFATc1 expression is elevated in prostate cancer and is an independent predictor of biochemical recurrence after radical prostatectomy. 103rd Annual Meeting United States & Canadian Academy of Pathology, March 2014, San Diego, California; *Mod Pathol* 27(Suppl 2): 239A, 2014.

3. Semenogelin I promotes prostate cancer cell growth via functioning an androgen receptor coactivator and protecting against zinc cytotoxicity. American Urological Association 109th Annual Meeting, May 2014, Orlando, Florida; *J Urol* 191(4 Suppl): e325, 2014.

4. Estrogen receptor alpha prevents bladder cancer via INPP4B/AKT pathway: In vivo knockout mouse evidence and human tissue analysis. American Urological Association 109th Annual Meeting, May 2014, Orlando, Florida; *J Urol* 191(4 Suppl): e362, 2014.

5. Nuclear factor of activated T-cells (NFAT) promotes prostate cancer progression. American Urological Association 109th Annual Meeting, May 2014, Orlando, Florida; *J Urol* 191(4 Suppl): e454, 2014.

6. Reduced glucocorticoid receptor expression predicts bladder tumor recurrence and progression. American Urological Association 109th Annual Meeting, May 2014, Orlando, Florida; *J Urol* 191(4 Suppl): e499, 2014.

7. Estrogen receptor α in cancer associated fibroblasts suppresses prostate cancer invasion via modulation of thrombospondin-2 and matrix metalloproteinase 3. American Urological Association 109th Annual Meeting, May 2014, Orlando, Florida; *J Urol* 191(4 Suppl): e584, 2014.

8. Ureteroscopy-assisted retrograde nephrostomy: A 150 case experience. American Urological Association 109th Annual Meeting, May 2014, Orlando, Florida; *J Urol* 191(4 Suppl): e903-e904, 2014.

9. The impact of androgen deprivation therapy on bladder cancer recurrence: Retrospective analysis. 6th European Multidisciplinary Meeting on Urological Cancers (EMUC), November 2014, Lisbon, Portugal; *Eur Urol Suppl* 13(5): 145, 2014.

7. Inventions, Patents, and Licenses

Nothing to report.

8. Reportable Outcomes

Nothing to report.

9. Other Achievements

- Promotion to Associate Professor of Pathology and Laboratory Medicine at the University of Rochester School of Medicine and Dentistry (November 1, 2011)

- Transfer to Johns Hopkins University School of Medicine as an Associate Professor (tenure-track) of Pathology and Urology (July 1, 2013)

- Other professional development activities (as a training award)
  Invited Talks/Conferences/Seminars:

12/2010	SUNY Upstate Medical University (Department of Urology Grand Rounds), Syracuse, New York
03/2011	University of Rochester (Department of Urology Grand Rounds), Rochester, New York
10/2011	Educational lecture at the 76th Annual Meeting of the Eastern Section of the Japanese Urological Association, Yokohama, Japan
10/2011	University of Occupational and Environmental Health (Department of Urology), Kitakyushu, Japan
10/2011	Mie Pathologists' Association, Tsu, Japan
10/2011	Yokohama City University Medical Association, Yokohama, Japan
04/2012	Johns Hopkins University (Department of Pathology), Baltimore, Maryland
03/2013	National Cancer Institute (Urologic Oncology Branch), Bethesda, Maryland
03/2013	Association of Japanese Life Scientists (Kinyokai) in the National Institutes of Health, Bethesda, Maryland
03/2013	Special Lecture at the 22nd Annual Meeting of the Japanese Society for Molecular and Cellular Urology, Kochi, Japan
03/2013	Special Lecture at the 15th Urological Genome Seminar, Kochi, Japan
03/2013	National Taiwan University Hospital (Department of Urology), Taipei, Taiwan
03/2013	Chang Gung Memorial Hospital (Genitourinary Oncology Tumor Board), Linkou, Taiwan
03/2013	National Taiwan University Hospital (Department of Pathology), Taipei, Taiwan
06/2013	Lecture for the Japanese Society of Urological Pathology at the 102nd Annual Meeting of the Japanese Society of Pathology, Sapporo, Japan
06/2014	Chang Gung University College of Medicine (Department of Urology), Kaohsiung, Taiwan
06/2014	Chang Gung Memorial Hospital at Kaohsiung Medical Center (Department of Pathology), Kaohsiung, Taiwan
06/2014	Educational lecture at the 55th Annual Spring Meeting of the Japanese Society of Clinical Cytology, Yokohama, Japan
09/2014	NIH/NCI (Medical Oncology Service), Bethesda, Maryland

11/2014   Johns Hopkins University (Department of Pathology Grand Rounds), Baltimore, Maryland

Advisory Committees, Review Groups/Study Sections:
2010   Scientific Review Panel (Endocrinology), Department of Defense Prostate Cancer Research Program – Idea Development Award
2011   Scientific Review Panel (Endocrinology & Immunology), Department of Defense Prostate Cancer Research Program – Idea Development Award
2012   External Reviewer, Michael Smith Foundation for Health Research, Canada
2012   Scientific Review Panel (Endocrinology), Department of Defense Prostate Cancer Research Program – Exploration-Hypothesis Development Award
2013   External Reviewer, Israel Science Foundation, Israel
2013   External Reviewer, University of California at Irvine Clinical and Translational Science Institute pilot project program
2013   Scientific Review Panel (Endocrinology), Department of Defense Prostate Cancer Research Program – Exploration-Hypothesis Development Award
2014   Scientific Review Panel (Endocrinology), Department of Defense Prostate Cancer Research Program – Idea Development Award
2014   External Reviewer, UK Medical Research Council DPFS/DCS Grants, United Kingdom

- Personnel who received pay from the research effort: 1) Koji Izumi, MD; 2) Hitoshi Ishiguro, PhD; 3) Takashi Kawahara, MD; and 4) Hiroki Ide, MD

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Tsai M-Y, Zhang Y, Yang L, Chang C: Interruption of nuclear factor κB signaling by the androgen receptor facilitates 12-*O*-tetradecanoylphorbolactate-induced apoptosis in androgen-sensitive prostate cancer LNCaP cells. *Cancer Res* 63: 7106-7112, 2003.

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11. Appendices

Reprints:
1. Kawahara T, Miyamoto H: Androgen receptor antagonists in the treatment of prostate cancer. *Clin Immunol Endocr Metab Drugs* 1(1): 11-19, 2014.
2. Canacci AM, Izumi K, Zheng Y, Gordetsky J, Yao JL, Miyamoto H: Expression of semenogelins I and II and its prognostic significance in human prostate cancer. *Prostate* 71(10): 1108-1114, 2011.
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10. Li Y, Zheng Y, Izumi K, Ishiguro H, Ye B, Li F, Miyamoto H: Androgen activates β-catenin signaling in bladder cancer cells. *Endocr-Relat Cancer* 20(3): 293-304, 2013.

11. Izumi K, Li Y, Ishiguro H, Zheng Y, Yao JL, Netto GJ, Miyamoto H: Expression of UDP-glucuronosyltransferase 1A in bladder cancer: association with prognosis and regulation by estrogen. *Mol Carcinogen* 54(4): 314-324, 2014.

12. Kawahara T, Kashiwagi E, Ide H, Li Y, Zheng Y, Ishiguro H, Miyamoto H: The role of NFATc1 in prostate cancer progression: Cyclosporine A and tacrolimus inhibit cell proliferation, migration, and invasion. *Prostate* 75(6): 573-584, 2015.

Androgen Receptor Antagonists in the Treatment of Prostate Cancer

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Abstract: Antiandrogens that block androgen action through the androgen receptor, often in conjunction with chemical or surgical castration, have been widely used for the treatment of advanced prostate cancer. Although this treatment produces a significant clinical response in most of the patients, the majority of the responders eventually develop recurrences termed castration-resistant prostate cancer. In addition, clinically available androgen receptor antagonists have been shown to possess agonist activity, resulting in an increase in serum prostate-specific antigen levels, which is known as the antiandrogen withdrawal syndrome. Recent studies have demonstrated that new types of androgen receptor signaling inhibitors improve survival in men with castration-resistant prostate cancer. Moreover, other drugs may have the potential of not inducing androgen withdrawal response. This article reviews the characteristics of classical and recent androgen receptor antagonists as well as their clinical efficacy in prostate cancer patients. Novel experimental compounds that may more specifically and effectively target androgens and/or androgen receptor signals in hormone-naive and possibly castration-resistant prostate cancer cells are also discussed.

Keywords: Androgen, androgen receptor, antiandrogen, castration, combined androgen blockade, cytochrome P450-17, hormonal therapy, prostate cancer.

INTRODUCTION

Prostate cancer is one of the most common causes of malignancy and cancer death among men worldwide [1]. Since the first report in 1941 [2], androgen deprivation therapy has contributed to the management of almost every stage of prostate cancer [3-7]. Hormonal manipulation in men with prostate cancer can be achieved by reduction in the availability of androgens and/or interference with their functions through the androgen receptor (AR) pathway. Thus, antiandrogens are often used in conjunction with castration as combined androgen blockade (CAB). However, after a brief clinical response, most of the responders ultimately develop hormone-refractory tumors known as castration-resistant prostate cancer (CRPC). Emerging evidence showing AR activation is often associated with its overexpression in CRPC [3-7] suggesting that hormone-refractory tumors remain AR-dependent for their growth. Accordingly, therapeutic options, including not only cytotoxic agents such as docetaxel but also novel AR signaling inhibitors, have been evaluated in patients with CRPC [8, 9]. In addition, classical antiandrogens that competitively inhibit binding of androgens to the AR in target cells have been reported to raise the levels of prostate-specific antigen (PSA), an AR-responsive gene, during CAB [10]. This phenomenon is known as antiandrogen withdrawal syndrome (AWS), and a subset of patients benefit from the withdrawal of antiandrogens. This article aims to provide clinical and molecular

evidence supporting the efficacy of classical and new AR antagonists in prostate cancer as well as its controversies.

STEROIDAL ANTIANDROGENIC DRUGS

Steroidal antiandrogens do not only compete with androgens for the binding to the AR but also contribute to a decrease in plasma levels of testosterone and 5α-dihydrotestosterone (DHT) by slowing the release of pituitary leutenizing hormone (LH) and by partial inhibition of 5α-reductase [4, 11]. Thus, steroidal antiandrogens, as single agents, may yield CAB. There are a few steroidal antiandrogens, including cyproterone acetate (CPA), megestrol acetate (MGA), and chlormadinone acetate (CMA), which have been clinically used for the treatment of prostate cancer as monotherapy or in combination with castration.

CPA

CPA was first reported in 1967 as a steroidal antiandrogenic agent that inhibited the action of adrenal and testicular androgens in prostatic cells [12]. It also possesses progestogenic activity, leading to a centrally mediated reduction in testicular secretion of androgens [13]. In earlier clinical studies of prostate cancer, there were no significant differences in disease-specific survival between CPA monotherapy and any other forms of androgen ablation, including surgical castration, LH-releasing hormone (LH-RH) agonists, and non-steroidal androgens [14]. In a more recent randomized clinical trial involving 310 men with metastatic prostate cancer, CPA monotherapy was again shown to have similar efficacy in disease progression and survival to a non-steroidal antiandrogen flutamide monotherapy [15]. A collaborative

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meta-analysis demonstrated a survival disadvantage (13% increase in the risk of death) with CPA in men with advanced prostate cancer who underwent CAB, compared to an advantage (8% decrease in the risk of death) with non-steroidal antiandrogens [16]. CPA has been associated with various adverse effects, such as cardiovascular events and other complications that may relate to the decline in testosterone levels, dyspnea, and occasional hepatotoxicity [17]. On the other hand, CPA can also be used to prevent castration-related hot flashes [18].

MGA

MGA is a synthetic progesterone derivative that possesses an antigonadotropic effect. It was often used as first- or second-line hormonal therapy in the late 1970s and early 1980s, however, only limited efficacy was seen. For instance, MGA monotherapy showed partial remission in 70% of patients with stage D prostate cancer with the median durations of response and survival of 10 and 20 months, respectively [19]. As second-line treatment for recurrent and metastatic prostate cancer, MGA showed little effects with 3.6% partial response and 25.0% stable disease [20]. Currently, the role of MGA treatment in prostate cancer patients might be limited to the management of hot flashes during androgen deprivation therapy. In a substantial portion of the patients long-term (*e.g.* 3 years) use of MGA was reported to be well tolerated [21].

CMA

CMA is another steroidal progestin that additionally shows antiandrogenic and antigonadotropic effects. It has been mainly used in Japan for the treatment of benign prostatic hyperplasia or prostate cancer [22, 23]. In a randomized multicenter study involving 151 men with untreated prostate cancer, addition of CMA to LH-RH agonist resulted in a significantly longer progression-free survival compared to LH-RH agonist monotherapy, while the two cohorts showed no difference in the rate of complete response defined by normalization of PSA levels [22]. In another prospective study comparing the efficacy of CAB between CMA and a non-steroidal antiandrogen flutamide in prostate cancers unfeasible for radical prostatectomy, there was no significant difference in response rate at 24 weeks of treatment (87.5% in the CMA group *vs.* 86.4% in the flutamide group) [23]. However, in this study [23], CMA was more favorable as to liver function as well as hot flashes and increases in testosterone levels several days after the first injection of a LH-RH analogue. Thus, like other steroidal antiandro-gens, CMA is likely effective for suppression of hot flashes during CAB. In a prospective study in patients undergoing CAB, CMA was found to more effectively prevent the occurrence of hot flashes and sweating compared with a non-steroidal antiandrogen bicalutamide [24].

1ST GENERATION NON-STEROIDAL ANTIANDRO-GENIC DRUGS

The first generation non-steroidal antiandrogenic agents include flutamide, nilutamide, and bicalutamide. In contrast to steroidal antiandrogens, these compounds show marginal effects on the inhibition of testicular androgen synthesis.

Numerous studies have assessed the efficacy of each non-steroidal antiandrogen as a component of CAB in the treatment of prostate cancer. In a meta-analysis of 27 prospective studies involving 8275 patients with advanced disease, no overall significant difference in the 5-year survival rate between castration monotherapy (23.6%) versus CAB with a steroidal or non-steroidal antiandrogen (25.4%) was observed [16]. However, as aforementioned, survival benefit was seen in CAB with non-steroidal antiandrogens (flutamide, nilutamide) [16]. A subsequent meta-analysis of 20 trials also showed a 5% improvement in survival at 5 years (30% *vs.* 25%) with CAB [25]. Thus, these studies as well as more recent data [26, 27] show a minimal advantage of CAB with non-steroidal antiandrogens in long-term survival over castration alone. In this section, the findings for non-steroidal antiandrogen monotherapy in prostate cancer are primarily discussed, although it appears to be less effective than castration alone or CAB [26]. There have been no comparative studies of the efficacy of different non-steroidal antiandrogens as monotherapy.

Flutamide Monotherapy

Flutamide was the first non-steroidal antiandrogen that was widely used as a component of CAB. A previous review summarized initial studies reporting the efficacy of flutamide as monotherapy (*i.e.* 68% of nearly 500 patients with untreated advanced prostate cancer showing a partial response), although most studies were relatively small and were not phase III trials [28]. A recent phase II trial demonstrated that 5 (25%) of 20 men who had biochemical (PSA) recurrence after definitive therapy for prostate cancer and received low-dose flutamide (125 mg twice daily) remained progression-free [29]. Subsequent studies comparing the efficacies of flutamide with or without orchiectomy showed no significant differences in the rate and duration of response between the two cohorts [30]. In a randomized study, diethylstilbestrol, a synthetic estrogen, was shown to prolong overall survival in men with stage D2 disease, compared to flutamide alone [31]. Elevations in amino-transferases, gynecomastia, and diarrhea have been noted during treatment with flutamide, and the rate of treatment withdrawal for drug-related adverse events was reported to be higher with flutamide than any other non-steroidal antiandrogens [28]. Flutamide-induced hepatotoxicity is occasionally severe and some patients develop fulminant hepatitis [32].

Nilutamide Monotherapy

There have been no randomized studies of nilutamide monotherapy reported. A small study involving 26 men with untreated metastatic prostate cancer showed responses to nilutamide monotherapy in 91% of the cases, with a median duration of overall survival of 23 months [33]. However, the survival rate in this study appeared to be less than that achieved by CAB with nilutamide [33]. A multicenter, randomized, clinical trial comparing nilutamide versus placebo after surgical castration revealed that addition of nilutamide produced significantly (p=0.0326) longer overall survival (27.3 months) than placebo group (23.6 months) [34]. Nonetheless, relatively high incidences of unique adverse effects, including visual problems (adverse light-dark adaptation), alcohol intolerance, and respiratory disturbance, have been

observed [35]. Additionally, nilutamide causes a higher incidence of nausea and vomiting than the other non-steroidal antiandrogens, while the incidence of diarrhea and gynecomastia is lower with nilutamide than flutamide [35]. These findings may have discouraged conducting larger trials using nilutamide.

Bicalutamide Monotherapy

Of available non-steroidal antiandrogens, bicalutamide as monotherapy has been most extensively studied. Early comparative trials showed better outcomes in patients with metastatic prostate cancer undergoing castration compared to bicalutamide monotherapy at 50 mg/day [36]. In contrast, subsequent studies with bicalutamide at 100 or 150 mg/day revealed equivalent efficacy between bicalutamide monotherapy and surgical/medical castration alone [37, 38] or CAB with flutamide [39] or nilutamide [40]. Bicalutamide at 150 mg/day has also been shown to have a more favorable side effect profile than flutamide and nilutamide [28, 39, 40]. More recent, large, randomized, prospective trials involving 8113 men demonstrated that bicalutamide at 150 mg/day, either as monotherapy or adjuvant to standard care (radical prostatectomy, radiotherapy, watchful waiting), improved progression-free survival in patients with locally advanced prostate cancer, but not in those with localized disease, and confirmed the quality of life benefit and tolerability of bicalutamide [41]. Thus, bicalutamide at 150 mg/day, either alone or as adjuvant therapy, has been considered an alternative for patients with locally advanced prostate cancer.

Alternative Antiandrogen Therapy

The clinical efficacy of alternative antiandrogen therapy has been reported, although the detailed mechanism remains unclear [42, 43]. For instance, in patients with CRPC who had received a trial of at least one antiandrogen, treatment with a high dose (150 or 200 mg/day) of bicalutamide, nilutamide, and CPA led to declines greater than 50% in PSA levels in 14-23%, 29-50%, and 4% of the cases, respectively [42]. Similarly, significant PSA decreases (50% or greater) in response to alternative antiandrogens after second-line hormonal therapy was started were seen in 83 (35.8%) of 232 patients, including 34.2% and 43.6% responders switching from bicalutamide to flutamide and from flutamide to bicalutamide, respectively [43]. Other responders included those with glucocorticoids (14-61%) and ketoconazole plus hydrocortisone (27-63%) [42]. However, the responses were often short-lived (*i.e.* 2-11 months).

Ketoconazole

Ketoconazole is a synthetic anti-fungal agent that non-specifically inhibits several enzymes, including 11β-hydroxylase and cytochrome P450-17 (CYP17), resulting in suppression of both testicular and adrenal androgen biosynthesis [44]. It has also been shown to compete weakly with androgens for AR binding [45] and may thus function as an AR antagonist. A number of clinical trials have been performed to assess the effect of ketoconazole as a second-line hormonal therapy for prostate cancer [46]. Phase II trials of second-line treatment showed that ketoconazole with hydrocortisone replacement significantly (>50%) reduced PSA levels in 31 to 63% of patients with median duration of re-

sponse lasting 3.5 to 7.5 months [42, 47, 48]. In a phase III randomized study for those who were resistant to first-line non-steroidal antiandrogen therapy, second-line ketoconazole following antiandrogen withdrawal resulted in PSA declines in 34 (27%) of 128 patients, compared to 11% (15 of 132 patients; p=0.002) following antiandrogen withdrawal alone [49]. However, in this trial median overall survival was not significantly different between the two groups [15.3 (ketoconazole) *vs.* 16.7 (antiandrogen withdrawal only) months; p=0.936]. In addition, because of low selectivity for CYP17 inhibition, high doses of ketoconazole are required, resulting in significant side effects including hepatotoxicity, gastrointestinal toxicity, and adrenal insufficiency [46].

2^(ND) GENERATION NON-STEROIDAL ANTIANDROGENIC DRUGS

As aforementioned, there are two types of resistance to hormonal therapy in patients with prostate cancer, AWS and CRPC.

AWS, first described in flutamide in 1993, is acknowledged as a general phenomenon of a PSA decrease, often with subjective or objective symptomatic improvement, on discontinuation of antiandrogens including bicalutamide, nilutamide, CPA, and CMA [10, 50, 51]. This phenomenon has been observed more often with non-steroidal antiandrogens. Many retrospective and several prospective studies have suggested that a significant number (15-80%) of patients treated with CAB display withdrawal responses as determined by PSA decline of more than 50%. Thus, a large portion of patients under CAB presenting with an increase in PSA level may benefit from discontinuing the antiandrogen before initiating second-line treatment. However, the duration of response is usually limited (4-8 months), and the patients subsequently develop CRPC. Molecular mechanisms responsible for AWS in which antiandrogens function as agonists are not completely understood yet include alterations (*i.e.* mutation, amplification) of *AR* gene and its co-regulatory proteins and activation of non-AR pathways (*e.g.* mitogen-activated protein kinase).

A variety of mechanisms are emerging that may be involved in the development of CRPC [3-7]. Nonetheless, the AR signaling pathway likely remains critical in most cancer cells from patients with clinically defined CRPC. The mechanisms include: 1) activation of AR transcription by, in addition to testosterone and DHT, adrenal androgens, progesterone, estrogens, and even antiandrogens; 2) AR variations and abnormalities changing the activity, for example, altering ligand specificity, which may lead to a "superactive" AR that responds to very low levels of androgens or other hormonal agents; and 3) AR activation by specific growth factors and cytokines in a ligand-independent manner.

The efficacy of the 2^(nd) generation non-steroidal antiandrogens has been being assessed in patients with androgen-sensitive prostate cancer as well as CRPC. The targets of these new drugs are depicted in (Fig. **1**).

Abiraterone Acetate (CB-7630)

Abiraterone is a selective inhibitor of CYP17 and is 10-30 fold more potent than ketoconazole [52]. In two phase I trials, mean half-life of abiraterone was found to range from
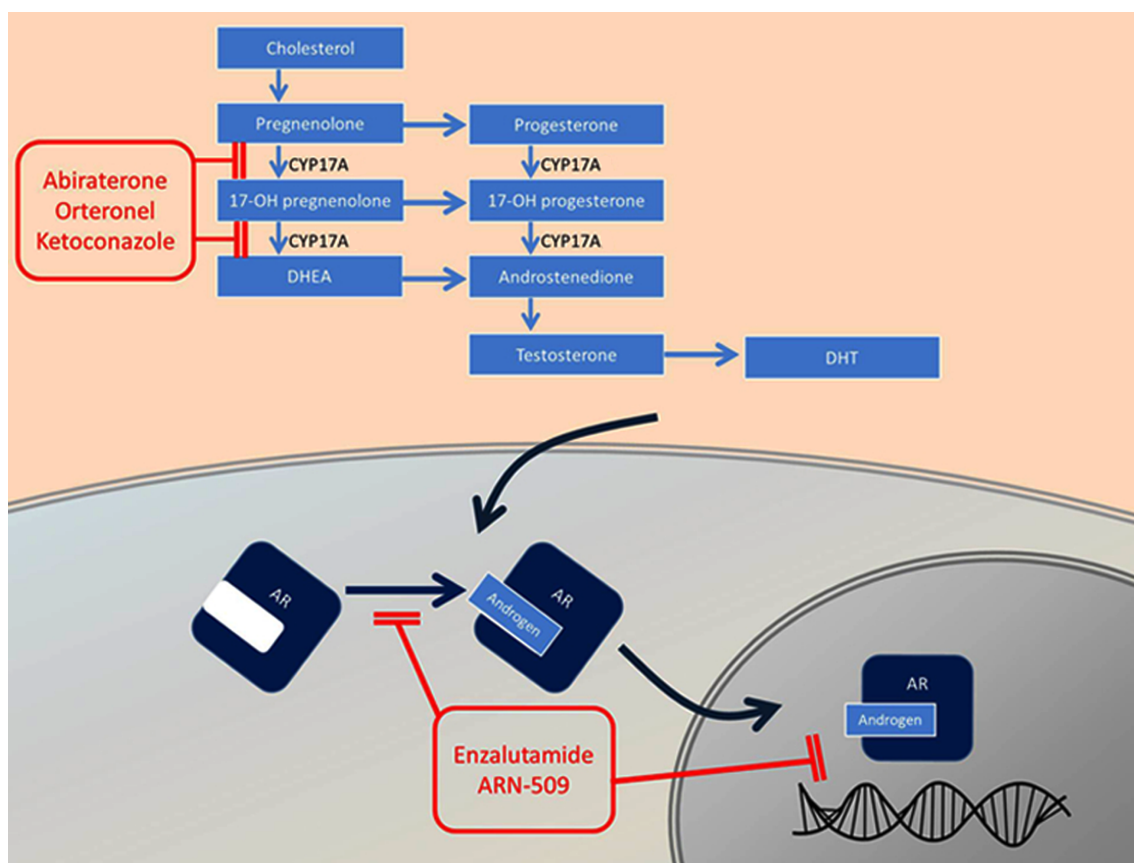
Fig. (1). Androgen synthesis pathway and therapeutic targets of 2nd generation antiandrogenic drugs.

5 to 14 hours and the time to Cmax ranged from 1.5 to 4 hours, suggesting that this inhibitor was likely safe and its optimal dose was 1000 mg/day [53, 54]. In these studies, the levels of testosterone and dehydroepiandrosterone (DHEA) were reduced from 7.4 ng/dL to less than 1 ng/dL and from 282.4 ng/dL to 83.6 ng/dL, respectively. Treatment with abiraterone alone increased adrenocorticotropic hormone and steroids upstream of CYP17, but the addition of dexamethasone resulted in decreases in their levels [53]. The efficacy of abiraterone in chemotherapy-naive men with metastatic CRPC has been assessed by phase I/II trials. In two of these studies [53, 54], PSA responses of at least 50% were observed in 55-57% of the patients. The PSA response was seen not only in ketoconazole-naive patients [8 of 14 (61%)] but also in patients who had previously received ketoconazole [10 of 19 (53%)] [54]. The combination of abiraterone with glucocorticoids has also been used in men with metastatic CRPC following chemotherapy. Two phase II studies in patients who received docetaxel-based therapy showed PSA decreases (more than 50%) in 24 (51%) out of 47 [55] and 22 (36%) out of 58 [56] patients. Similarly, in the latter study, the response was still seen even in ketoconazole-pretreated patients (26%) [56]. These findings were confirmed by subsequent placebo-controlled randomized phase III studies of abiraterone in metastatic CRPC with [57] or without [58] prior chemotherapy. In both studies, abiraterone was shown to significantly improve progression-free and overall survivals. The former study [57] showing longer overall survival (15.8 months in the abiraterone group *vs.*

11.2 months in the placebo group; p<0.0001) and higher PSA response rate (29.5% *vs.* 5.5%; p<0.0001) recently led to the FDA approval of abiraterone for the treatment of chemotherapy-refractory CRPC. Unlike ketoconazole, abiraterone is usually well-tolerated, yet its side effects primarily related to secondary mineralocorticoid excess included fluid retention or edema, hypokalemia, and hypertension [53-58]. Cardiac disorders and abnormalities on liver function tests were also more commonly seen in patients with abiraterone plus a glucocorticoid than in those with placebo/glucocorticoid alone [57, 58].

Enzalutamide (MDV-3100)

Enzalutamide is an oral AR signaling inhibitor that not only blocks androgen binding to AR, with a 5-8 fold higher affinity compared to bicalutamide, but also prevents AR nuclear translocation, DNA binding, and coactivator recruitment [59]. The half-life was about one week (3 to 13 days) and the time to maximum concentration was between 30 minutes to 4 hours [60]. The first phase I/II study revealed 43% (13 of 30 patients) had PSA declines of 50% or greater [59]. In another important phase I/II study, 140 men with CRPC received increasing doses of enzalutamide (30–600 mg) and more than half of the patients had PSA declines (greater than 50%) [60]. The maximum tolerated dose of enzalutamide was determined to be 240 mg, but treatment with higher doses (>150 mg) did not provide additional antitumor activity. The rate of patients with the PSA response at

12 weeks was significantly higher in the chemotherapy-naive group (57%) than in those with prior chemotherapy (36%, p<0.02) and in the ketoconazole-naive group (71%) than in those with prior ketoconazole therapy (37%, p=0.0007), but not in those who received two or fewer previous hormone treatments (61%) compared to three or more previous hormones (50%). A subsequent international, randomized, double-blind, phase III study involving 1199 CRPC patients who received prior docetaxel chemotherapy showed that enzalutamide at a dose of 160 mg/day (*vs.* placebo) significantly improved the rate of PSA declines of more than 50% (54% *vs.* 2%, p<0.001), median overall survival (18.4 *vs.* 13.6 months, p <0.001), and all other end points (*e.g.* soft tissue response, quality-of-life response, time to PSA progression, radiographic progression-free survival, time to the first skeletal-related event) [61]. Enzalutamide at a dose of 160 mg/day was recently approved by the FDA for the treatment of metastatic CRPC following docetaxel-containing chemotherapy. Additional randomized clinical trials are currently under way to evaluate enzalutamide in patients with, for instance, localized prostate cancer and chemotherapy-naive CRPC. The most common adverse event included dose-dependent fatigue followed by nausea, diarrhea, musculoskeletal pain, hot flashes, and headache [60, 61].

Orteronel (TAK-700)

Orteronel is a non-steroidal inhibitor of 17,20-lyase activity of CYP17A1 [62]. In monkey models, orteronel administered orally successfully reduced serum levels of DHEA and testosterone [62]. An initial phase I/II trial established the safety of orteronel and showed its efficacy in patients with metastatic CRPC comparable to that of abiraterone [63]. Orteronel treatment also resulted in a considerable decrease in the number of circulating tumor cells [63]. Despite more selective inhibition of 17,20-lyase, orteronel showed side effects similar to those seen in abiraterone therapy. An ongoing randomized, double-blind, multicenter, phase III trial is evaluating orteronel plus prednisone (*vs.* placebo plus prednisone) in men with metastatic CRPC that has progressed following docetaxel-based therapy.

ARN-509

ARN-509, isolated using structure activity-relationship (SAR)-guided medical chemistry as a non-steroidal antiandrogen that retains full antagonist activity, has the structure similar to enzalutamide but greater *in vivo* activity in CRPC xenograft models [64]. In prostate cancer cells overexpressing AR, ARN-509 binds to the AR with 7- to 10-fold greater affinity than bicalutamide and inhibits AR nuclear translocation and DNA binding leading to tumor regression and apoptosis [64]. Similarly to enzalutamide that could induce seizure in animals by an off-target mechanism, ARN-509 was found *in vitro* to inhibit GABA-A currents. However, relatively low central nervous system penetration of ARN-509 has suggested a lower risk of drug-induced seizure [65]. A phase I study demonstrated that ARN-509 was well tolerated and PSA declines of more than 50% were achieved in 55% of patients with CRPC [66]. Phase II clinical trials of ARN-509 are being performed in patients with non-metastatic CRPC, metastatic CRPC, and abiraterone-refractory CRPC.

NEWER COMPOUNDS AROUND ANTIANDROGENS

Galeterone (TOK-001, VN/124-1)

Galeterone was synthesized as a powerful inhibitor of both human and rat testicular CYP17. It disrupts androgen signaling pathways simultaneously via mechanisms involving inhibitions of CYP17, competitive binding of androgens and AR, AR nuclear translocation, and AR protein expression and thereby strongly inhibits the growth of human prostate cancer *in vitro* and *in vivo* [67, 68]. Preliminary results of a phase I study included rare severe adverse events with galeterone and its suppressive activity in 49 men with chemotherapy-naive CRPC (*i.e.* 49% with ≥30% PSA reduction, 22% with ≥50% PSA reduction) [69].

BMS-641988

BMS-641988 is a small-molecule AR antagonist with a 20-fold higher binding affinity to AR and has greater potency as an inhibitor of AR-mediated transcription and tumor growth in *in vitro* and *in vivo* prostate cancer models, compared with bicalutamide [70]. In particular, BMS-641988 showed anti-tumor activity in a model resistant to bicalutamide treatment. In addition, global gene expression analysis has shown that treatment with BMS-641988 results in a phenotype closer to that achievable with castration rather than that with bicalutamide treatment. A phase I study of 61 men with CRPC revealed one patient who developed an epileptic seizure and 10 (16%) who had PSA declines of greater than 30% [71]. Antiandrogen withdrawal response was also seen in several patients.

EPI-001

EPI-001 is a small-molecule that specifically binds to the activation function-1 region and thereby inhibits transactivation of amino-terminal domain of the AR without interacting with the ligand-binding domain (LBD) [72]. Thus, EPI-0001 inhibits constitutively active AR lacking the LBD. Consistent with its suppression of AR activity in prostate cancer cells, EPI-0001 slows inhibitory effects on AR-mediated cell proliferation *in vitro* and tumor growth in mouse xenograft models with no apparent toxicity [72].

Nicotinamides

A chemical library screening identified a lead compound, 6-(3,4-dihydro-*1H*-isoquinolin-2-yl)-*N*-(6-methylpyridin-2-yl)nicotinamide (DIMN), targeting AR, although its AR binding activity was found to be lower than that of flutamide or bicalutamide [73]. DIMN inhibited androgen-induced AR transactivation and the proliferation of prostate cancer cells. A subsequent SRA study of DIMN analogues offered the structural optimization of nicotinamides as candidates of potent AR antagonists [74]. A few compounds indeed showed stronger inhibitory effects on prostate cancer growth *in vitro*, especially in AR-positive/androgen-independent cells, compared to those of DIMN.

Pyrrole Derivatives

During the screening of their compound library, Yamamoto *et al.* found that a pyrrole derivative exhibited antago-

nistic activity against wild-type AR as well as mutant ARs detected in prostate cancer cells/tissues where flutamide or bicalutamide functions as an agonist [75]. SAR analyses of 4-phenylpyrrole derivatives further isolated compounds showing anti-tumor activities in mouse xenograft models for bicalutamide-resistant prostate cancer.

RB346

Screening of a series of synthetic DHT derivatives identified several compounds possessing antagonistic activities with marginal agonist effects [76]. Of the DHT derivatives,

RB346 showed the strongest antagonistic effects and inhibited DHT-induced proliferation and PSA expression in prostate cancer cells. One unique characteristic of RB346 included the flexibility of its side chain which enabled to bind different mutated LBDs of the AR with high affinity.

Thiohydantoin Derivatives

A thiohydantoin derivative, CH4933468, was initially found to completely block androgen-induced AR transcription [77]. It also inhibited the proliferation of androgen-sensitive prostate cancer cells as well as those having AR
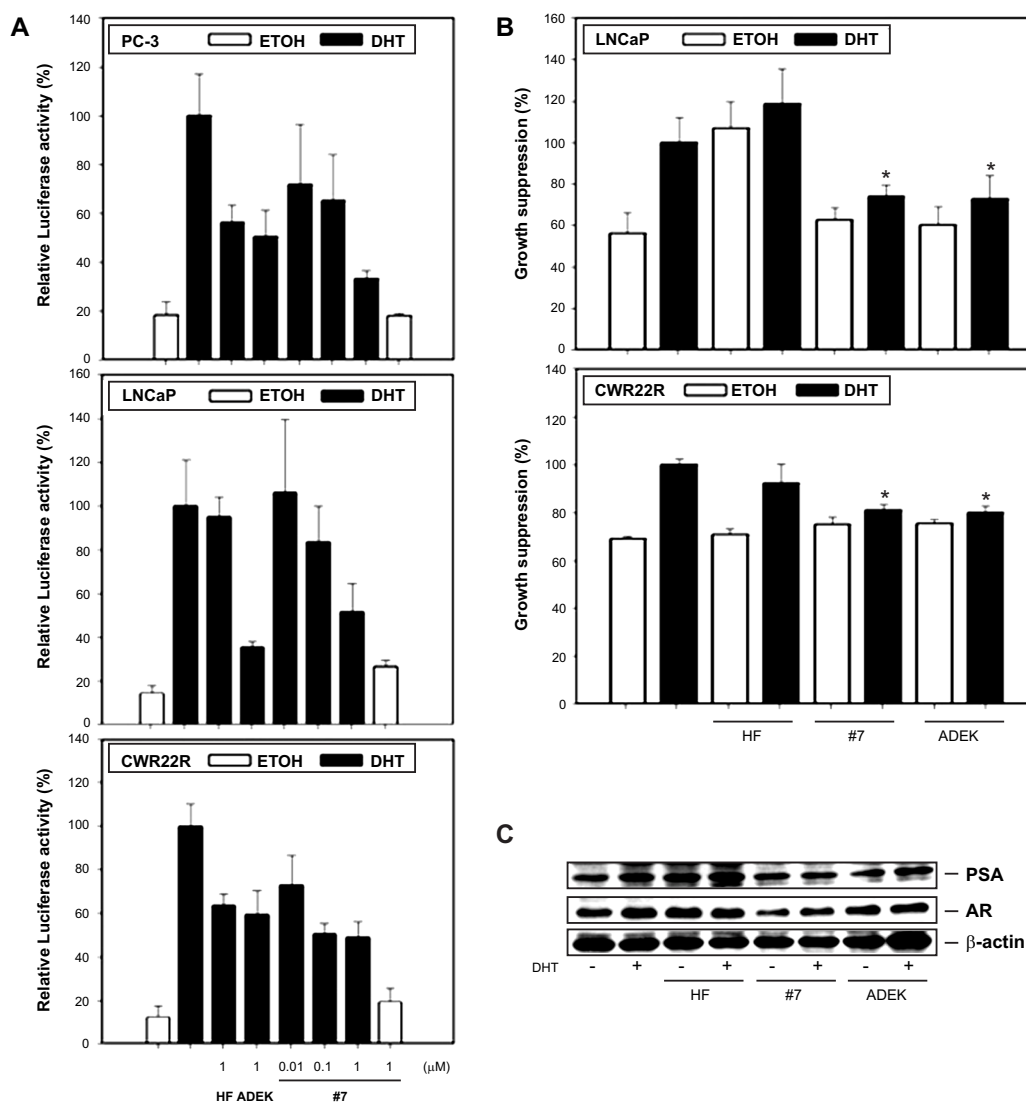
Fig. (2). The effects of DHEA derivatives. (**A**) AR transcriptional activity was determined in PC-3, LNCaP, or CWR22Rv1 cells transfected with wild-type AR (PC-3 only) and a MMTV-luciferase reporter plasmid and cultured for 24 hours with 1 μM HF, 1 μM ADEK, or various concentrations of PM-VI-7 (#7) in the absence (ETOH; white bars) or presence (black bars) of 1 nM DHT. The luciferase activity is presented relative to that in the presence of DHT in each panel (second lanes; set as 100%). Values represent the mean ± SD of at least three determinations. (**B**) Cell viability was measured in LNCaP or CWR22Rv1 cells cultured for 96 hours with 1 μM HF, 1 μM ADEK, or 1 μM #7 in the absence (ETOH; white bars) or presence (black bars) of 1 nM DHT. The MTT assay was performed and growth induction/suppression is presented relative to cell number with DHT treatment in each panel (second lanes; set as 100%). Values represent the mean ± SD of at least three determinations. *$p<0.05$ (*vs.* DHT). (**C**) The expression of PSA and AR was assessed in cell extracts from LNCaP cultured for 48 hours with 1 μM HF, 1 μM ADEK, or 1 μM #7 in the absence or presence of 1 nM DHT. Western blotting was then performed, using an antibody to PSA (upper), AR (middle), or β-actin (lower). The 33 kDa (for PSA) and 110 kDa (for AR) proteins were detected. β-Actin expression (43 kDa) was used as an internal control.

overexpression and being hypersensitive to low levels of androgens *in vitro*. Subsequent screening of other thiohydantoin derivatives revealed that CH5137291 exhibited antitumor activity in CRPC xenografts where CH4933468 failed to prevent their growth [78].

Arabilin

A novel compound, arabilin, with two known structural isomers, spectinabilin and SNF4435C, produced by *Streptomyces* sp. MK756-CF1, was shown to compete the binding of DHT to AR and inhibit DHT-induced PSA mRNA expression [79]. Spectinabilin showed approximately 100-fold potent suppressive effects on PSA expression compared with arabilin and SNF4435C while the precise mechanism for the inhibitions remains unclear.

Synthetic DHEA Derivatives

We previously found that androgens largely derived from the adrenal gland have intrinsic androgenic activity which was not completely antagonized by two antiandrogens, hydroxyflutamide (HF) and bicalutamide [80, 81]. We have then hypothesized that DHEA metabolites or their synthetic derivatives are able to bind to the AR with low, if any, agonist activity and thus function as improved AR antagonists. Initial screening of DHEA derivatives identified several compounds, including 3β-acetoxyandrost-1,5-diene-17-ethylene-ketal (ADEK) and 3β-hydroxyandrosta-5,16-diene (HAD), which showed only marginal agonist effects and suppressed significantly androgen-induced AR transcriptional activity in prostate cancer cells [82-84]. We also showed that these compounds had an affinity for the AR, allowing a competition with androgens for binding, and inhibited DHT-mediated prostate cancer cell growth and its PSA expression [82, 83]. Thus, ADEK and HAD seemed to have the potential to function as potent antiandrogens that carry fewer risks of withdrawal response if used for therapy in prostate cancer patients. However, the disadvantage of using these compounds in clinical settings included their estrogenic activity [82, 83]. These most likely induce chemical castration which may still be beneficial to such patients, but could also cause severe side effects, such as cardiovascular toxicity. Another steroid derivative, 3-oxo-androst-1,4-diene-17-ethylene-ketal (OAK), showed anti-DHT effects on AR transcription and prostate cancer cell growth, with marginal estrogenic activity [83]. However, OAK at high concentrations (*e.g.* ≥0.5 µM) had androgenic activity similar to that of HF, especially in the presence of AR coactivators. Further screening of DHEA derivatives isolated a compound (PM-VI-7) that showed marginal androgenic/estrogenic activities as well as significant inhibitory effects on androgen-mediated transactivation of wild-type/mutant ARs, cell proliferation, and PSA expression (Fig. **2**; Miyamoto *et al.*, unpublished data). Of note, in contrast to HF, bicalutamide, and other DHEA derivatives, PM-VI-7 reduced AR protein expression in the presence or absence of androgens in prostate cancer cells (Fig. **2F**).

CONCLUSION

Classical antiandrogens, often in conjunction with castration, remain useful for the treatment of androgen-dependent prostate cancer. However, in this setting AWS can be induced by any of these drugs. In addition, none of these are effective for CRPC where the AR pathway is often activated. Recent evidence indicates that a new class of antiandrogens targeting AR (*e.g.* enzalutamide, ARN-509), CYP17 (*e.g.* abiraterone, orteronel), or both (*e.g.* galeterone) show efficacy for CRPC yet do not usually result in the cure of the disease. Some other investigational agents also show significant inhibitory effects on the growth of CRPC. Moreover, AR antagonists combined with other cytotoxic drugs have been being investigated in CRPC patients. To further improve outcomes of patients with advanced prostate cancer, we need to determine the way to prolong the androgen-dependent state and, more importantly, precise molecular mechanisms for the emergence of CRPC. The latter may lead to the development of novel treatment options for CRPC. Nonetheless, mechanisms are likely diverse among cases, and it is also possible that no single mechanism is utilized in every case. Therefore, it may be necessary to explore more individualized approaches.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

LIST OF ABBREVIATIONS

AR	=	Androgen receptor
ADEK	=	3β-acetoxyandrost-1,5-diene-17-ethylene-ketal
AWS	=	Antiandrogen withdrawal syndrome
CAB	=	Combined androgen blockade
CMA	=	Chlormadinone acetate
CPA	=	Cyproterone acetate
CRPC	=	Castration-resistant prostate cancer
CYP17	=	Cytochrome P450-17
DHEA	=	Dehydroepiandrosterone
DHT	=	5α-dihydroxytestosterone
DIMN	=	6-(3,4-dihydro-*1H*-isoquinolin-2-yl)-*N*-(6-methylpyridin-2-yl)nicotinamide
HAD	=	3β-hydroxyandrosta-5,16-diene
HF	=	Hydroxyflutamide
LBD	=	Ligand-binding domain
LH	=	Leutenizing hormone
LH-RH	=	Leutenizing hormone-releasing hormone
MGA	=	Megestrol acetate
OAK	=	3-oxo-androst-1,4-diene-17-ethylene-ketal
PSA	=	Prostate-specific antigen
SAR	=	Structure activity-relationship

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Expression of Semenogelins I and II and Its Prognostic Significance in Human Prostate Cancer

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BACKGROUND. Little is known about the role of semenogelins, seminal plasma proteins that play critical roles in semen clotting and subsequent liquefaction in the presence of zinc and prostate-specific antigen, in human malignancies.

METHODS. We investigated the expression of semenogelins in four human prostate cancer lines by RT-PCR and Western blotting as well as in 70 radical prostatectomy specimens by immunohistochemistry. Effects of semenogelin overexpression on prostate cancer cell proliferation were also assessed.

RESULTS. mRNA/protein signals for semenogelins I (SgI) and II (SgII) were detected only in androgen-sensitive LNCaP cells cultured with zinc. Transfection of SgI/SgII increased/decreased cell growth of androgen receptor (AR)-positive/semenogelin-negative CWR22Rv1 in the presence of zinc, whereas it showed marginal effects in AR-negative/semenogelin-negative PC-3 and DU145. Immunohistochemical studies showed that SgI and SgII stain positively in 55 (79%) and 31 (44%) cancer tissues, respectively, which was significantly higher than in corresponding benign tissues [SgI-positive in 13 (19%) cases ($P < 0.0001$) and SgII-positive in 15 (21%) cases ($P = 0.0066$)]. Among the histopathological parameters available for our patient cohort, there was an inverse association only between Gleason score (GS) and SgII expression (GS $\leq$ 7 vs. GS $\geq$ 8: $P = 0.0150$; GS7 vs. GS $\geq$ 8: $P = 0.0111$). Kaplan–Meier and log-rank tests further revealed that patients with SgI-positive/SgII-negative tumor have the highest risk for biochemical recurrence ($P = 0.0242$).

CONCLUSIONS. These results suggest the involvement of semenogelins in prostate cancer and their prognostic values in predicting cancer progression after radical prostatectomy. Additional functional analyses of semenogelins are necessary to determine their biological significance in prostate cancer. *Prostate 71: 1108–1114, 2011.* © 2010 Wiley-Liss, Inc.

KEY WORDS: **seminal plasma proteins; immunohistochemistry; prostate-specific antigen; zinc; biochemical recurrence**

INTRODUCTION

Prostate cancer has been a leading cause of cancer-related death among men [1,2]. Although radical prostatectomy can offer the possibility of cure of localized prostate cancer, a substantial number of patients will develop recurrent disease following the surgery [3–5]. Clinical outcomes in prostate cancer have been strongly correlated with histopathological factors (e.g., Gleason grade, stage, surgical margin status) as well as numerous biomolecules [4,5]. Nonetheless, these markers remain insufficient to precisely predict the potential for recurrence or metastasis. Further controversy includes the selection of appropriate

patients who will benefit from hormonal therapy immediately after a definitive treatment [2,6].

Semenogelins, seminal plasma motility inhibitors predominantly secreted by the seminal vesicle, are the main structural components of human semen coagulum. Semenogelin I (SgI), a $\sim$50 kDa protein, and semenogelin II (SgII), a $\sim$63 kDa protein, are encoded by two homologous genes located 11.5 kb apart in the chromosome 20 q12–q13.1 regions, and as such, share 78% of their amino acid sequence [7–9]. Semen spontaneously coagulates upon ejaculation thereby trapping sperm. Semenogelins inhibit sperm motility; however, within minutes, the coagulum liquefies thus releasing motile sperm. This process is hastened by kallikrein-related peptidase 3, also known as prostate-specific antigen (PSA), which targets and degrades semenogelins into lower molecular mass (5–20 kDa) fragments [9–11]. Semenogelins regulate the activity of PSA in that they initiate their own degradation by chelating Zn^{2+}, which normally acts to inhibit the protease activity of PSA [12]. Thus, physiological functions of semenogelins in male reproductive organs, in conjunction with zinc and PSA, have been thoroughly studied.

In addition to expression in the seminal vesicle and other male genital organs, immunoreactivity to semenogelins has been demonstrated in non-genital organs, such as the trachea, salivary gland, pancreas, kidney, and retina [13,14], suggesting a physiological role of these proteins as modulators of zinc-dependent proteases throughout the body [10,12]. Semenogelins have also been detected in human malignancies, including lung carcinomas [15], melanoma [15], and leukemias [16]. Expression of semenogelins in genitourinary cancers has not been extensively studied, but Lundwall et al. [13] detected mRNAs of SgI and SgII in an androgen-sensitive prostate cancer cell line LNCaP. This study also demonstrated semenogelin immunoreactivity in a single case of human prostate cancer, using an antibody that recognizes both SgI and SgII. The purpose of this study was to further elucidate semenogelin expression and its potential role as a biomarker in prostate cancer.

MATERIALS AND METHODS

Cell Culture

The human prostatic adenocarcinoma cell lines, LNCaP, CWR22Rv1, PC-3, and DU145, were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and cultured in the presence of zinc chloride (Sigma, St. Louis, MO) for 48 hr prior to isolation of RNA or protein.

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Total RNA was extracted, using Trizol reagent (Invitrogen, Carlsbad, CA), from the human seminal vesicle specimen and the prostate cancer cell lines cultured with 100 µM zinc for 48 hr. Isolated RNA was reverse transcribed to cDNA, using Omniscript (Qiagen, Valencia, CA) with oligo-dT-primer. Subsequent PCR was performed, using Advantage 2 PCR kit (Clontech, Mountain View, CA), as previously described [13]. PCR conditions included an initial denaturation at 95°C for 1 min, 40 cycles consisting of 95°C for 30 sec and 68°C for 1 min, and an extra incubation at 68°C for 1 min. The following primers were used: SgI, 5′-GCAGACACCAACATGGATCT-CA-3′ and 3′-CTGAGGTCAACTGACACCTTGA-5′; SgII, 5′-AGCATGAGGTTGCCCAAGATGA-3′ and 3′-GAGGTCGGGTGACACCTTGC-5′; and GAPDH, 5′-CTCCTCCACCTTTGACGCTG-3′ and 3′-CATACC-AGGAAATGAGCTTGACAA-5′. Reaction products were analyzed by electrophoresis in a 2.5% agarose gel containing ethidium bromide.

Western Blotting

To detect semenogelin proteins in prostate cancer cells cultured with 100 µM zinc for 48 hr, Western blotting was performed, as described previously [17] with some modifications. Briefly, equal amounts of protein (50 µg) obtained from cell extracts were separated in 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electroblotting, using a standard protocol. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (Bio-Rad) for 1 hr at room temperature and then immunoblotted with each primary antibody [SgI, diluted 1:1,000, Abcam (Cambridge, MA); SgII, diluted 1:1,000, Abcam; or β-actin, diluted 1:1,000, Santa Cruz Biotechnology (Santa Cruz, CA)] for 2 hr at room temperature, followed by incubation with respective secondary antibodies for 1 hr at room temperature. Antibody–protein complexes were visualized using an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL) and Kodak X-OMAT film (Eastman Kodak, Rochester, NY). As positive controls, CWR22Rv1 cells transfected with semenogelin expression plasmids (pSG5-SgI and pSG5-SgII by inserting full-length SgI and SgII, respectively), according to GeneJuice transfection instructions (Novagen, Gibbstown, NJ), were used.

Cell Proliferation Assay

We used the MTT (thiazolyl blue) assay to assess cell growth, as described previously [17] with minor modifications. Cells (3×10^3) seeded in 96-well tissue culture plates were first transfected with pSG5, pSG5-SgI, or pSG5-SgII, as described above, and then cultured in medium supplemented with 10% FBS in the presence or absence of 100 µM zinc. After 4 days of treatment, we added 10 µl of MTT (Sigma) stock solution (5 mg/ml) to each well with 0.1 ml of medium for 4 hr at 37°C. Then, we replaced the medium with 100 µl of DMSO, incubated for 5 min at room temperature, and measured the absorbance at a wavelength of 570 nm with background subtraction at 655 nm.

Tissue Samples and Immunohistochemical Staining

Appropriate approval from the Institutional Review Board of the University of Rochester Medical Center was obtained prior to construction and use of the tissue microarray (TMA). Prostate TMA was constructed from 70 formalin fixed paraffin embedded prostatectomy tissue specimens retrieved from the Surgical Pathology archives, using 1.0 mm cores of representative benign and tumor lesions. The mean age of the patients at presentation was 60.2 years (range: 42–78 years) and the mean follow-up after the surgery was 29.2 months. None of the patients had received therapy with hormonal reagents, radiation, or other anticancer drugs pre- or post-operatively prior to clinical or biochemical recurrence. Biochemical recurrence was defined as a single PSA level of ≥ 0.2 ng/ml. TMA sections (4 µm thick) were immunohistochemically labeled, using the same primary antibodies to SgI (diluted 1:1,000, Abcam) and SgII (diluted 1:1,000, Abcam) as utilized for Western blotting, performed on the automated staining system, as described previously [18]. Appropriate positive controls (human seminal vesicle tissue) were run concurrently. As a negative control, sections were treated in an identical fashion except for replacing the primary antibody with non-immune rabbit IgG. German Immunoreactive Score (0–12) was calculated, separately in benign and malignant glands, by multiplying the percentage of immunoreactive cells (0% = 0; 1–10% = 1; 11–50% = 2; 51–80% = 3; 81–100% = 4) by staining intensity (negative = 0; weak = 1; moderate = 2; strong = 3). Scores were considered negative (0–1), weakly positive (2–4), moderately positive (6–8), and strongly positive (9–12).

Statistical Analyses

Differences in cell growth in the two groups were analyzed by Student's *t*-test. Differences in semenogelin expression in prostate TMA were analyzed by Fisher's exact test or Chi-square test. Survival rates in patients were calculated by the Kaplan–Meier method and comparison was made by log-rank test. Multivariate analysis was then performed with the Cox proportional hazards regression model. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Expression in Prostate Cancer Cell Lines

We first examined the expression of SgI and SgII in human prostate cancer cells by RT-PCR. As depicted in Figure 1A, mRNA signals for SgI and SgII were detected in androgen receptor (AR)-positive LNCaP cells cultured in the presence of zinc. The levels of their expression were lower when no zinc was added in culture media (data not shown). No semenogelin transcripts were detected in other tested lines, including CWR22Rv1, PC-3, and DU145 cells. Western blot analysis was also performed to determine if these prostate cancer cell lines express semenogelin proteins.
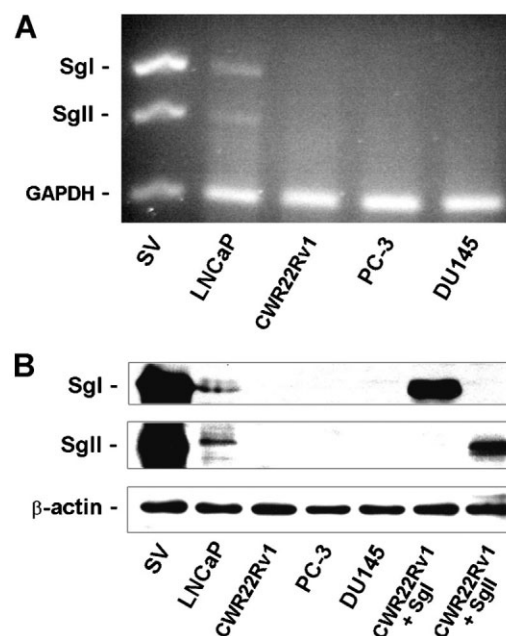

Fig. 1. Expression of SgI and SgII in human prostate cancer cell lines. **A**: Transcripts were amplified by RT-PCR and separated by electrophoresis for SgI (180 bp) and SgII (130 bp). Human seminal vesicle (SV) served as the positive control. PCR products derived from GAPDH mRNA (71 bp) served as the internal control. **B**: Cell extracts were analyzed on Western blots, using an antibody to SgI (upper), SgII (middle), or β-actin (lower). Human seminal vesicle (SV) and CWR22Rv1 cell line transfected with a semenogelin expression plasmid (pSG5-SgI or pSG5-SgII) served as the positive controls. The 52 kDa (for SgI) and 65 kDa (for SgII) proteins were detected, as indicated. β-Actin expression (43 kDa) served as the internal control.

Correlating with their mRNA expression, SgI and SgII proteins were detected only in LNCaP cells (Fig. 1B).

Effects on Prostate Cancer Cell Proliferation

We next performed the MTT assay to evaluate the effects of semenogelins on cell growth of prostate cancer lines. SgI or SgII was expressed in CWR22Rv1, PC-3, and DU145 that were then cultured for 4 days in the presence or absence of additional zinc. As expected, 100 μM of zinc significantly inhibited the growth of AR-positive CWR22Rv1 cells (Fig. 2; $P < 0.0001$). In the presence of zinc, transfection of SgI significantly increased the growth of CWR22Rv1 cells by 12% ($P = 0.0137$), whereas SgII overexpression decreased it by 14% ($P = 0.1696$). In contrast, SgI and SgII only marginally affected the growth of CWR22Rv1 cells in the absence of additional zinc. In addition, overexpression of semenogelins, with or without zinc, showed marginal effects (i.e., <5%) on the growth of AR-negative PC-3 and DU145 cells, although zinc significantly inhibited the growth of these cells (figure not shown).

Immunoreactivity in Prostate Cancer Tissue Samples

We then performed immunohistochemical stains for SgI and SgII in 70 radical prostatectomy specimens. Positive signals were detected predominantly in nuclei of epithelial cells (Fig. 3). Cytoplasmic or luminal staining is also seen in some cases of carcinoma glands. The results of semenogelin expression in tissue samples are summarized in Table I.
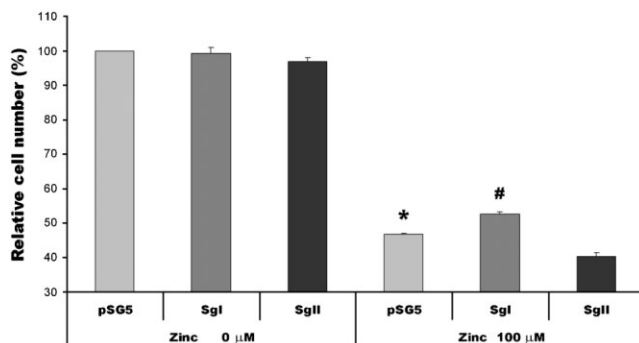

Fig. 2. Cell growth of the prostate cancer line overexpressing SgI or SgII. CWR22Rv1 cells transfected with either pSG5 (vector only), pSG5-SgI, or pSG5-SgII were cultured in medium supplemented with 10% FBS in the presence or absence of 100 μM zinc for 4 days, as indicated. Proliferation was assayed with MTT, and growth induction or suppression is presented relative to cell number with pSG5 vector transfection/no zinc treatment (first lane; set as 100%). Each value represents the mean + SD of at least three determinations. *$P < 0.0001$ (vs. pSG5 vector transfection/0 μM zinc). #$P = 0.0137$ (vs. pSG5 vector transfection/100 μM zinc).
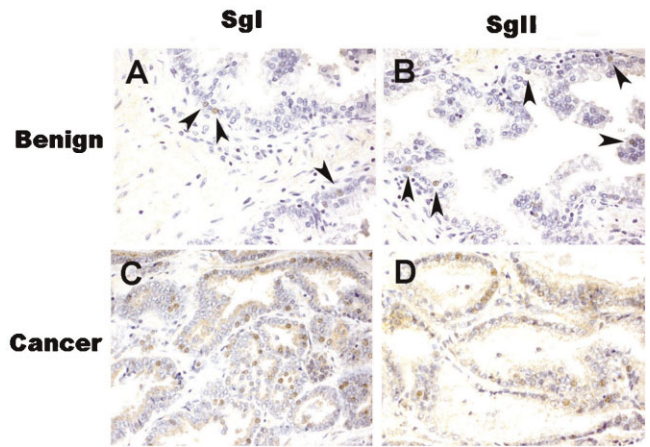
Fig. 3. Immunohistochemistry of SgI and SgII in benign and malignant prostate tissue. Occasional positive signals (arrowheads) of SgI (**A**) or SgII (**B**) were observed in benign epithelial cells but stronger, more diffuse staining of SgI (**C**) and SgII (**D**) was seen in carcinoma cells. Original magnification 200×.

Overall, SgI was positive in 13 (19%; all weak) benign tissues and in 55 (79%; 53 weak and 2 moderate) cancer tissues. Similarly, SgII was positive in 15 (21%; all weak) benign tissues and in 31 (44%; all weak) cancer tissues. Thus, the expression of SgI ($P < 0.0001$) or SgII ($P = 0.0066$) was significantly higher in carcinoma cells than in non-malignant epithelial cells. Higher scores of SgI and SgII were seen in two and nine cases, respectively, in benign glands, compared to cancer glands. Seventy cases included 28 SgI-positive/SgII-positive tumors, 27 SgI-positive/SgII-negative tumors, 3 SgI-negative/SgII-positive tumors, and 12 SgI-negative/SgII-negative tumors. Associations between SgI expression and SgII expression in prostate cancer were statistically significant ($P = 0.0234$). Among 55 SgI-positive cases, 28 (51%) were also SgII-positive, whereas 28 (90%) of 31 SgII-positive cases were SgI-positive.

Semenogelin Expression and Clinicopathologic Features

We evaluated the associations between the expression of semenogelin and histopathological features available for our patient cohort. No significant correlation between SgI expression and Gleason score (GS) was observed. In contrast, it was noted that SgII expression negatively correlates with GS (≤ 7 vs. ≥ 8: $P = 0.0150$; 7 vs. ≥ 8: $P = 0.0111$; ≤ 6 vs. ≥ 8: $P = 0.0674$; ≤ 6 vs. ≥ 7: $P = 0.8095$). There were no statistically significant correlations between staining and other histopathological parameters analyzed. In 14 patients with extraprostatic extension, SgI and SgII were positive in 12 (86%; all weak) and 7 (50%; all weak)

TABLE I. Expression of SgI and SgII in 70 Tumor and Benign Prostate Tissue Microarrays

	SgI				SgII			
	Negative (0–1)	Weak (2–4)	Moderate/ strong (6–12)		Negative (0–1)	Weak (2–4)	Moderate/ strong (6–12)	
Cancer (n = 70)	15	53	2	P < 0.0001	39	31	0	P = 0.0066
Benign (n = 70)	57	13	0		55	15	0	
Gleason score				P > 0.10				P < 0.05
≤6 (n = 26)	4	20	2		15	11	0	
7 (n = 37)	10	27	0		17	20	0	
≥8 (n = 7)	1	6	0		7	0	0	
Extraprostatic extension (pT3a)				P > 0.10				P > 0.10
Negative (n = 56)	13	41	2		33	23	0	
Positive (n = 14)	2	12	0		7	7	0	
Seminal vesicle involvement (pT3b)				P > 0.10				P > 0.10
Negative (n = 66)	14	50	2		36	30	0	
Positive (n = 4)	1	3	0		3	1	0	
Surgical margin status				P > 0.10				P > 0.10
Negative (n = 61)	13	46	2		33	28	0	
Positive (n = 9)	2	7	0		6	3	0	
Lymph node metastases				P > 0.10				P > 0.10
Negative (n = 45)	8	37	0		22	23	0	
Positive (n = 2)	0	2	0		0	2	0	

tumors, respectively. In four patients with seminal vesicle involvement, SgI and SgII were positive in three (75%; all weak) and one (25%; weak) tumors, respectively. In nine patients with positive surgical margins, SgI and SgII were positive in seven (78%; all weak) and three (33%; all weak) tumors, respectively. Additionally, both SgI and SgII were weakly positive in two patients with lymph node metastases (out of 47 cases with pelvic lymph node dissection).

To assess possible associations between semenogelin staining and disease recurrence, we then performed Kaplan–Meier analysis coupled with log-rank test. Of the 70 patients with a mean follow-up of 29.2 months, 6 (8.6%) had a clinical or biochemical recurrence after radical prostatectomy. Of these, five (83%) cases exhibited GS ≥ 8, extraprostatic extension, seminal vesicle involvement, positive surgical margins, and/or lymph node metastasis. SgI alone (Fig. 4A; $P = 0.5409$) or SgII alone (Fig. 4B; $P = 0.2378$) showed no strong correlation with recurrence. Nonetheless, there were trends to weakly associate between SgI positivity or SgII negativity and a risk of recurrence. Interestingly, patients with SgI-positive/SgII-negative tumor had a significantly higher risk of recurrence (Fig. 4C), compared to those with SgI-positive/SgII-positive or SgI-negative tumor ($P = 0.0242$), SgI-positive/SgII-positive tumor ($P = 0.1087$), or SgI-negative tumor ($P = 0.2102$). Multivariate analysis revealed that semenogelin
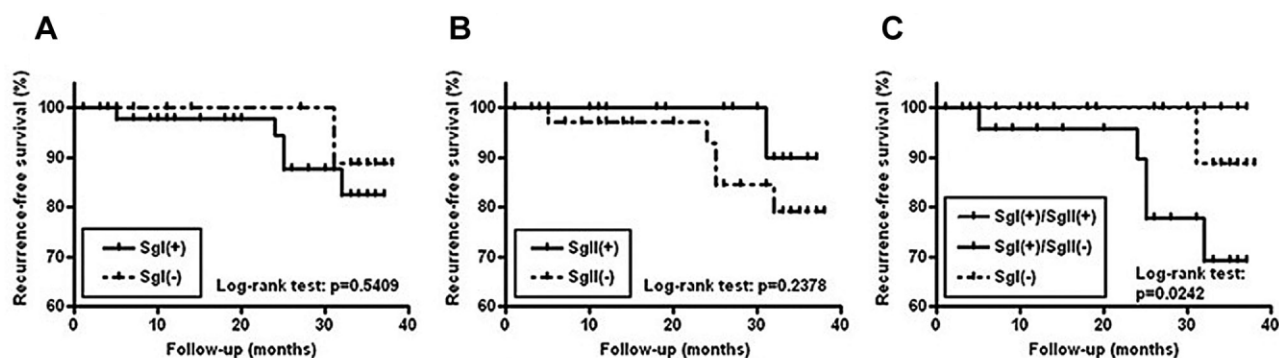

Fig. 4. Kaplan–Meier analysis of recurrence-free survival according to SgI expression (**A**), SgII expression (**B**), or both (**C**). Biochemical recurrence was defined as a single PSA level of ≥0.2 ng/ml.

expression, as well as each analyzed variable, is not an independent prognostic factor ($P > 0.05$) in our cohort.

DISCUSSION

Compared with well-recognized physiological functions in male reproductive system [7–13], the role of semenogelins in human malignancies is poorly understood. To our knowledge, there have been only a few studies published, showing the expression of semenogelins in lung carcinomas [15], melanoma [15], and leukemias [16]. In prostate cancer, another study demonstrated the expression of SgI and SgII in an androgen-sensitive cell line LNCaP, but not in androgen-insensitive cell lines DU145 (with a faint SgII signal) and PC-3, by RT-PCR, as well as in a single case of tissue specimen by immunohistochemistry [13]. In the current study, we confirmed these findings in prostate cancer cell lines both in mRNA and protein levels. We additionally showed that another AR-positive prostate cancer CWR22Rv1 cells are negative for SgI and SgII. Interestingly, the addition of zinc in culture medium increases mRNA expression of SgI and SgII in LNCaP cells. The prostate contains the highest level of zinc of any soft tissue and its concentrations in prostate cancer, although a significant decrease is seen, remain much higher than those in other tissue or blood plasma [19,20]. Nonetheless, experimental evidence suggests that high zinc levels prevent prostate carcinogenesis, although it is controversial whether zinc supplements indeed decrease the risk of prostate cancer [20–22]. Furthermore, zinc shows an inhibitory effect on cell growth of prostate cancer [23,24]. Thus, zinc may exhibit contradictory effects on prostate cancer. Semenogelins have been known to bind Zn^{2+}, abundant in semen, to function as a regulator of PSA activity [12]. Accordingly, functional analysis of semenogelins in prostate cancer may provide not only an explanation for the conflicting results on zinc and prostate cancer but also potential therapeutic targets.

A pilot experiment was performed to see if semenogelins affect prostate cancer cell proliferation. Semenogelins showed marginal effects on the growth of AR-negative/semenogelin-negative PC-3 and DU145 cells in the presence or absence of additional zinc. Remarkably, co-expression of SgI and SgII resulted in an increase and a decrease, respectively, in the growth of AR-positive/semenogelin-negative CWR22Rv1 cells only in the presence of a high level of zinc. These results suggest that semenogelins may require both zinc and AR to function as modulators of prostate cancer cell proliferation. In these assays, however, we used a transient transfection method to co-express respective semenogelins, and generally low transfection efficiency might have obscured the effect of

semenogelins on cell growth. As discussed above [23,24], zinc was confirmed to inhibit the growth of all the three prostate cancer lines examined. Further studies of semenogelins, in conjunction with zinc, androgen, AR, and PSA, each of which is deeply involved in prostate carcinogenesis and cancer progression, remain necessary.

As noted, Lundwall et al. [13] included only one case of prostate cancer in their immunohistochemical analysis. This is the first study that extensively elucidates semenogelin immunoreactivity in prostate cancer. There were highly significant differences in the expression of SgI and SgII between carcinoma and corresponding benign tissues. Most of SgII-positive tumors were SgI-positive, whereas roughly half of SgI-positive tumors were SgII-positive. Thus, strong correlations between expressions of SgI and SgII in prostate cancer were observed. These results suggest the involvement of semenogelins in prostate cancer development.

Among the histopathological parameters available for our patient cohort, GS showed an inverse association with SgII expression, but not with SgI expression. There were no significant associations between semenogelin expression and other parameters, including extraprostatic extension, seminal vesicle invasion, surgical margin status, and lymph node metastasis. The present study also analyzed and compared the prognostic value of semenogelin expression, using Kaplan–Meier survival curves and log-rank test. The expression status of either SgI alone or SgII alone did not strongly correlate with recurrence. It is noteworthy that a combination of semenogelins showed statistically significant differences: patients with SgI-positive/SgII-negative tumor had the highest risk of recurrence. However, the significance of this result may need to be taken into consideration with low recurrence rate (n = 6), possibly due to the relatively short follow-up duration (mean of 29.2 months) in our cohort of radical prostatectomy patients. This data, along with the results of our cell proliferation assay, suggests that SgI may promote prostate cancer progression and SgII may protect against it. In spite of numerous attempts, no reliable biomarkers for accurate prediction of prostate cancer recurrence besides preoperative PSA value and GS have been identified [4,5]. SgI and SgII could be such markers, yet further combinations with other potential markers may lead to identifying more independent prognostic predictors.

CONCLUSIONS

The expression and prognostic significance of SgI and SgII in prostate cancer were investigated. Our results may indicate that both SgI and SgII contribute to

prostate cancer development. Moreover, SgI and SgII are suggested to have contradictory effects on prostate cancer progression. Further studies including larger patient cohorts with longer follow-up are needed to validate these initial results. Additional functional analyses of semenogelins in prostate cancer are also necessary to determine their biological significance.

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European Association of Urology

Letter to the Editor NOT referring to a recent journal article

Eppin Expression in Prostate Cancer

Little is known about the biological functions of seminal plasma proteins in prostate cancer (PCa). We recently showed increased expression of semenogelins I (SgI) and II (SgII), which play critical roles in semen clotting, in PCa [1]. Our data also suggested that SgI promoted PCa cell proliferation, and its positivity correlated with tumor recurrence. SgI is bound to eppin (epidermal protease inhibitor), which is specifically expressed and secreted in the testis and epididymis, in seminal plasma and on the surface of spermatozoa, resulting in inhibition of sperm motility [2]. Indeed, antieppin antibodies have been evaluated as a form of male contraception [3]. In contrast, the role of eppin in neoplastic conditions in male genital organs, including prostatic adenocarcinoma, is poorly understood.

We immunohistochemically investigated the expression of eppin (H-100 rabbit polyclonal antibody, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the 70 radical prostatectomy specimens where semenogelin expression was assessed [1]. Appropriate positive controls (human epididymal tissue) and negative controls (primary antibody with nonimmune rabbit immunoglobulin G) were used. German Immunoreactive Score (0–12) was calculated separately in benign and malignant glands, as described [1]. Positive signals were detected predominantly in cytoplasm of epithelial/carcinoma cells (Fig. 1a). Nuclear or luminal staining is also seen in some cases. Additionally, secreted materials inside benign glands often showed a strong

immunoreactivity. Overall, eppin stained positively in 67 cancer tissues (95.7%; 1+: 37 cases; 2+: 25 cases; 3+: 5 cases; mean score [standard deviation (SD)]: 4.09 [$\pm$2.04]), which was significantly higher (Fisher exact test: $p = 0.0080$; student t test: $p = 0.0058$) than in corresponding benign tissues (56 positive cases [80.0%]; 1+: 39 cases; 2+: 13 cases; 3+: 4 cases; mean score [SD]: 3.14 [$\pm$2.09]).

We then evaluated the associations of eppin expression (ie, positivity, intensity, score) with histopathologic features available for our patient cohort. Scores (mean) tended to be higher in tumors with less aggressive characteristics (all $p > 0.05$): Gleason score $\leq$6 (4.33) vs 7 (4.08) vs $\geq$8 (3.21); pT2 (4.23) vs pT3a (3.46) vs pT3b (4.38); negative surgical margins (4.22) vs positive surgical margins (4.07); and N0 (4.02) vs N1 (5.00). No significant correlations between expressions of eppin and SgI (55 positives) or SgII (31 positives) were observed. Kaplan-Meier and log-rank tests in 70 patients with a mean follow-up of 29.5 mo further revealed that those with eppin-negative tumor, although there were only three cases, had a significantly higher risk of biochemical recurrence ($p = 0.0005$) (Fig. 1b). Notably, none of the five patients with eppin 3+ tumor had recurrence.

We demonstrated that eppin is overexpressed in PCa, and this likely correlates with more favorable prognosis. Although eppin is physiologically present in a protein complex containing SgI [2], strong association of their expression was not confirmed in PCa. Further studies including larger patient cohorts with longer follow-up are necessary to validate these initial results. The growth of PCa

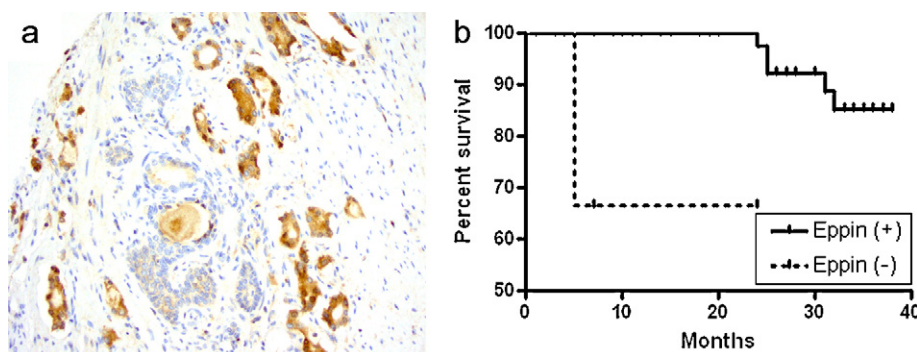Fig. 1 – Eppin expression and its relation to tumor recurrence: (a) Strongly positive eppin immunoreactivity was detected in prostate cancer glands but not in nonmalignant glands (present at the center). Original magnification × 200; (b) Kaplan-Meier analysis of recurrence-free survival according to eppin expression. Biochemical recurrence was defined as a single prostate-specific antigen level of $\geq$0.2 ng/ml.

is largely dependent on androgen receptor activity [4], and the eppin gene possesses a functional androgen response element [5]. Therefore, functional analyses of eppin, together with semenogelins, in PCa will be interesting to determine their biological significance.

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Dihydrotestosterone upregulates the expression of epidermal growth factor receptor and ERBB2 in androgen receptor-positive bladder cancer cells

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Abstract

Androgen receptor (AR) signals play important roles in bladder carcinogenesis and tumor progression. Activation of the epidermal growth factor receptor (EGFR) family, including EGFR and ERBB2, leads to bladder cancer cell growth and correlates with poor patients' prognosis. However, cross talk between AR and EGFR/ERBB2 pathways in bladder cancer remains poorly understood. In AR-positive bladder cancer UMUC3 and TCC-SUP cells, dihydrotestosterone (DHT) increased the expression of EGFR and ERBB2 both in mRNA and in protein levels, and an anti-androgen hydroxyflutamide antagonized the effect of DHT. The necessity of AR was confirmed by silencing the receptor, using short hairpin RNA (shRNA), in UMUC3 cells, as well as by expressing the receptor in AR-negative 5637 cells. Of note were much higher basal levels of EGFR and ERBB2 in UMUC3-control-shRNA than in UMUC3-AR-shRNA and those of EGFR in 5637-AR than in 5637-V. DHT additionally upregulated the levels of phosphorylation of EGFR (pEGFR) and its downstream proteins AKT (pAKT) and ERK1/2 (pERK), induced by EGF treatment, in AR-positive cells. Immunohistochemistry on cystectomy specimens showed strong associations between expressions of AR and EGFR ($P=0.0136$), pEGFR ($P=0.0041$), ERBB2 ($P=0.0331$), or pERK ($P=0.0274$), but not of pAKT ($P=0.5555$). The Kaplan–Meier and log-rank tests further revealed that positivity of AR ($P=0.0005$), EGFR ($P=0.2425$), pEGFR ($P=0.1579$), ERBB2 ($P=0.2997$), or pERK ($P=0.1270$) and negativity of pAKT ($P=0.0483$) were associated with tumor progression. Our results indicate that AR activation upregulates the expression of EGFR and ERBB2 in bladder cancer cells. AR signals may thus contribute to the progression of bladder cancer via regulation of the EGFR/ERBB2 pathways.

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Introduction

Urinary bladder cancer is the fourth most commonly diagnosed malignancy in males in the United States, accounting for 6.7% of all cancer cases (Jemal *et al.* 2010). However, in females, the morbidity of bladder cancer is much lower, accounting for 2.4% of all cancer cases. The classical concept has been tempting to attribute the sex-related differences in the risk of bladder cancer to environmental or lifestyle factors, such as industrial chemicals and cigarette smoke. Nonetheless, excess risk of bladder cancer in men

persisted after controlling for these carcinogenic factors (Hartge *et al.* 1990, Jemal *et al.* 2010). We have recently shown molecular evidence for the discrepancy indicating that androgen receptor (AR) signaling pathway is involved in bladder cancer (Miyamoto *et al.* 2007, Johnson *et al.* 2008). Castrated male and wild-type female mice had a lower incidence of bladder cancer induced by a chemical carcinogen than wild-type male mice. In addition, AR knockout completely prevented mice from bladder cancer development. We and others also showed that

androgens increased cell proliferation of AR-positive bladder cancer lines *in vitro* and *in vivo*, and that anti-androgen treatment or downregulation of AR abolished the effect of androgens (Miyamoto *et al.* 2007, Johnson *et al.* 2008, Boorjian *et al.* 2009, Wu *et al.* 2010). Thus, AR signals likely promote bladder carcinogenesis as well as cancer progression.

It is well known that in a variety of malignancies, activation of the epidermal growth factor receptor (EGFR) family, including EGFR and ERBB2, contributes to tumorigenesis and tumor progression. In bladder cancer, frequent overexpression and/or gene amplification of *EGFR/ERBB2* have been reported, which correlates with higher tumor grade/stage and poorer clinical outcome (Neal *et al.* 1990, Lipponen & Eskelinen 1994, Orlando *et al.* 1996, Miyamoto *et al.* 2000, Jimenez *et al.* 2001, Latif *et al.* 2004). Indeed, the EGFR pathway has been shown to play a critical role in cell proliferation, apoptosis, differentiation, migration, and angiogenesis in bladder cancer (Bellmunt *et al.* 2003, MacLaine *et al.* 2008). Ultimately, the efficacy of targeted therapy with novel agents directed at EGFR signaling pathway has been assessed in bladder cancer (Bellmunt *et al.* 2003, Latif *et al.* 2004, Black *et al.* 2007, Bhuvaneswari *et al.* 2009).

The cross talk between AR and EGFR/ERBB2 signaling pathways has been investigated in prostate and breast cancers (Culig *et al.* 1994, Scher *et al.* 1995, Craft *et al.* 1999, Signoretti *et al.* 2000, Di Lorenzo *et al.* 2002, Liu *et al.* 2005, Mukherjee & Mayer 2008, Naderi & Hughes-Davies 2008, Pignon *et al.* 2009). Specifically, in prostate cancer, EGFR and ERBB2 were overexpressed during the progression to hormone-independent state (Craft *et al.* 1999, Signoretti *et al.* 2000, Di Lorenzo *et al.* 2002). AR signals regulated *EGFR* and *ERBB2* gene expression in prostate cancer cells. Conversely, activation of EGFR and ERBB2 in prostate cancer cells lead to modulation of AR functions. Activated EGFR and ERBB2 could increase transactivation of AR in prostate cancer cells, which may be mediated by activation of their downstream proteins, mitogen-activated protein kinase (MAPK) and phosphatidyl-inositol 3-kinase (PI3K)/AKT, and subsequently an increased expression or phosphorylation of some transcriptional cofactors (Orio *et al.* 2002, Gregory *et al.* 2004, Mellinghoff *et al.* 2004).

In contrast, little is known about the relationship between AR and EGFR family in bladder cancer. The purpose of this study was to investigate the involvement of androgens/AR signaling in the EGFR/ERBB2 pathways in bladder cancer cells.

Materials and methods

Cell culture and chemicals

Human urothelial carcinoma cell lines UMUC3, TCC-SUP, and 5637, and human embryonic kidney cell line 293T (all obtained from the American Type Culture Collection, Manassas, VA, USA) were maintained in appropriate medium (Mediatech, Manassas, VA, USA; DMEM for UMUC3, TCC-SUP, and 293T; RPMI-1640 for 5637) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS at least 18 h before experimental treatment. We obtained dihydrotestosterone (DHT) and EGF from Sigma; hydroxyflutamide (HF) from Schering (Kenilworth, NJ, USA); cycloheximide from MP Biomedical (Solon, OH, USA); and PD168393 from Calbiochem (San Diego, CA, USA).

Stable cell lines with AR and AR-short hairpin RNA

To establish a stable AR knockdown cell line, UMUC3 directly transfected with a retrovirus vector pMSCV/U6-AR-short hairpin RNA (shRNA) or pMSCV/U6-control-shRNA, using GeneJuice transfection reagent (Novagen, Gibbstown, NJ, USA), was selected using 3 µg/ml puromycin (Sigma), as described previously (Miyamoto *et al.* 2007). In addition, a full-length wild-type human AR cDNA was subcloned into PWPI plasmid (Addgene, Cambridge, MA, USA), and the lentivirus-based vector (PWPI-AR/PWPI-control) with pMD2.G packaging and psPAX2 envelope plasmids (lentivirus:packaging:envelope = 2:1:1) was co-transfected into 293T cells, using GeneJuice. After 48 h of transfection, the target cells (5637) were cultured in the presence of viral supernatant containing 8 µg/ml polybrene (Millipore, Billerica, MA, USA) for 6 h. Flow cytometry was used to obtain pure cell line overexpressing AR (5637-AR) or vector only (5637-V).

Reporter gene assay

Bladder cancer cells at a density of 50–60% confluence in 24-well plates were co-transfected with 250 ng of MMTV-luc reporter plasmid DNA and 2.5 ng of PRL-TK-luc plasmid DNA, using GeneJuice, as described previously (Miyamoto *et al.* 2003). After 6 h of transfection, the medium was replaced with another medium supplemented with charcoal-stripped FBS in the presence or absence of ligands (DHT, HF, or both) for 24 h. Cells were harvested, lysed, and

assayed for luciferase activity, which was determined using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) and luminometer (TD-20/20, Turner BioSystems, Sunnyvale, CA, USA).

Cell proliferation assay

We used the MTT (methyl thiazolyl diphenyl tetrazolium bromide) assay to assess cell viability, as described previously (Canacci *et al.* 2011). Cells (3×10^3) seeded in 96-well tissue culture plates were incubated with medium supplemented with charcoal-stripped FBS containing ethanol or ligands (DHT and/or HF). The media were refreshed every other day. After 4 days of treatment, we added 10 µl MTT (Sigma) stock solution (5 mg/ml) to each well with 0.1 ml of medium for 4 h at 37 °C. Then, we replaced the medium with 100 µl DMSO, incubated for 5 min at room temperature, and measured the absorbance at a wavelength of 570 nm with background subtraction at 655 nm.

Reverse transcription and real-time PCR

Total RNA (0.5 µg) isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) was reverse transcribed using 1 µmol/l oligo (dT) primers and 4 units of Omniscript reverse transcriptase (Qiagen, Valencia, CA, USA) in a total volume of 20 µl. Real-time PCR was then performed in 15 µl system by using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously (Miyamoto *et al.* 2007). The primer sequences are given as below: EGFR (forward, 5′-CCAAGGCACGA-GTAACAA-3′; reverse, 5′-ACATAACCAGCCACCT-CC-3′); and ERBB2 (forward, 5′-TGACACCTAGC-GGAGCGAT-3′; reverse, 5′-GGGGGATGTGTTTT-CCCTCAA-3′). GAPDH (forward, 5′-CTCCTCCA-CCTTTGACGCTG-3′; reverse, 5′-CATACCAGGAA-ATGAGCTTGACAA-3′) was used as an internal control.

Western blot

Protein extraction and western blot were performed as described previously (Miyamoto *et al.* 2003) with minor modifications. Briefly, equal amounts of protein (50 µg) obtained from cell extracts were separated in 10% SDS–PAGE and transferred to polyvinylidene difluoride membrane (Millipore) by electroblotting using a standard protocol. Specific antibody binding was detected, using HRP detection system (Super-Signal West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, IL, USA). Anti-AR (N20) (diluted 1:1000), anti-EGFR (diluted 1:100), anti-ERBB2 (diluted 1:100), anti-ERK1/2 (diluted 1:1000), and anti-β-actin (diluted 1:1000) antibodies

were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphorylation of EGFR (pEGFR) (Tyr1068) (diluted 1:1000), anti-AKT (diluted 1:1000), anti-pAKT (Ser473) (diluted 1:1000), and anti-pERK1/2 (Thr202/Tyr204) (diluted 1:1000) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

Bladder tissue microarray and immunohistochemistry

Appropriate approval from the Institutional Review Board of the University of Rochester Medical Center was obtained prior to construction and use of the tissue microarray (TMA). Bladder cancer TMA was constructed from 24 formalin-fixed paraffin-embedded cystectomy tissue specimens retrieved from the Surgical Pathology archives, using 1.0 mm cores of representative tumor lesions. These 24 specimens were obtained from 19 men and 5 women, with a mean age at cystectomy of 66.2 years (range 49–86 years) and a mean follow-up after the surgery of 8.3 months (range 3–20 months). All the cases were histologically diagnosed as high-grade urothelial carcinoma. These included 2 pTis, 3 pT1, 4 pT2, and 15 ≥ pT3 tumors, and 12 node-negative and 12 node-positive tumors.

Immunohistochemical staining was performed as described previously (Miyamoto *et al.* 2007, Canacci *et al.* 2011) with minor modifications. Briefly, TMA sections (4 µm thick) were deparaffinized in xylene, rehydrated in a graded ethanol series, and incubated in 3% hydrogen peroxide to block endogenous peroxidase. Slides were incubated overnight at 4 °C with the same antibodies as utilized for western blot: anti-AR (diluted 1:100), anti-EGFR (diluted 1:50), anti-pEGFR (diluted 1:100), anti-ERBB2 (diluted 1:50), anti-pAKT (diluted 1:100), and anti-pERK1/2 (diluted 1:100). We then incubated the samples with a broad spectrum secondary antibody (Invitrogen). After being rinsed in PBS, the slides were incubated with diaminobenzidine (Invitrogen), and finally counterstained with hematoxylin. These stains were manually scored by one pathologist (H M) blinded to patient identity. German Immunoreactive score (0–12) was calculated, only in tumor cells, by multiplying the percentage of immunoreactive cells (0%, 0; 1–10%, 1; 11–50%, 2; 51–80%, 3; 81–100%, 4) by staining intensity (negative, 0; weak, 1; moderate, 2; strong, 3). Scores were considered as follows: negative (0–1) versus positive (2–12) for AR; negative (0–8) versus positive (for overexpression) (9–12) for EGFR/ERBB2; and negative (0–4) versus positive (6–12) for pEGFR/pAKT/pERK.

Statistical analyses

Differences in variables with a continuous distribution across dichotomous categories (i.e. luciferase activity, estimated cell proliferation) were analyzed by Student's *t*-test. Differences in protein expression between the two groups from human tissue samples were analyzed by Fisher's exact test or χ^2 test. Progression-free survival rates in patients were calculated by the Kaplan–Meier method, and comparison was made by log-rank test. *P* values <0.05 were considered to be statistically significant.

Results

Androgen mediates AR transactivation and cell proliferation in bladder cancer

It was found that two bladder cancer cell lines UMUC3 and TCC-SUP express AR (Miyamoto *et al*. 2007, Boorjian *et al*. 2009). We additionally showed that androgens increased AR transcriptional activity and cell proliferation in these two cell lines, and an anti-androgen HF antagonized the effects of androgens (Miyamoto *et al*. 2007). Using a reporter gene assay, we first confirmed the functional activity of AR in another bladder cancer cell line 5637 with a full-length wild-type AR stably expressed by lentivirus. Luciferase activity was determined in the cell extracts with transfection of a plasmid (MMTV-luc) containing an androgen response element (ARE) as a reporter of AR-mediated transcriptional activity and treatment of DHT and/or HF. As shown in Fig. 1A, DHT treatment increased luciferase activity to more than 70-fold in 5637-AR over mock treatment, and HF showing partial agonist activity (approximately sixfold) could block DHT-induced AR transcriptional activity. In AR-negative 5637-V cells, the basal activity with mock treatment was ∼55% of that in 5637-AR ($P=0.016$), and DHT and HF showed only marginal effects on AR transcription. We next performed the MTT assay to evaluate the effects of androgen on cell proliferation of AR-positive and AR-negative bladder cancer cell lines. As shown in Fig. 1B, in 5637-AR cells, DHT increased cell growth by ∼60% in 4 days (lane 1 vs 2, $P=0.003$) and HF at least partially blocked the DHT effect (lane 2 vs 4). In contrast, DHT and/or HF only marginally affected the growth of 5637-V cells (lane 7 vs 8–10). Additionally, using a specific inhibitor of EGFR, PD168393, we assessed the contribution of the EGFR pathway to DHT-induced growth stimulation. As expected, PD168393 reduced the cell growth by ∼73% (Fig. 1B, lane 1 vs 5, $P<0.001$). In the presence of PD168393, the growth induction rate by
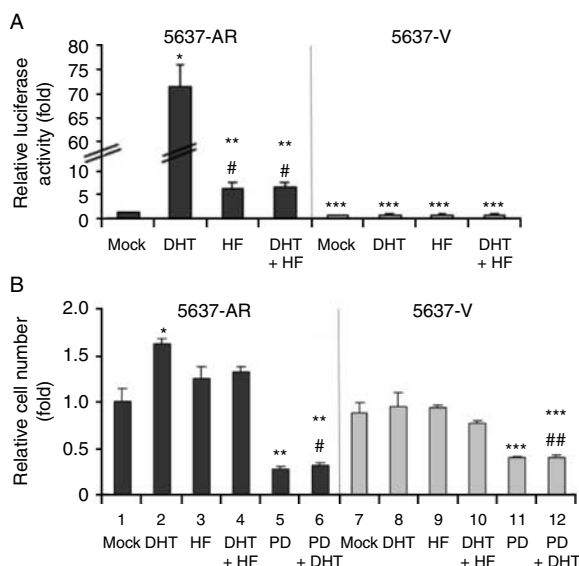

Figure 1 Effects of androgen on AR transactivation (A) and cell viability (B) in bladder cancer lines. (A) 5367-AR or 5637-V cells transfected with MMTV-ARE-luc were cultured for 24 h in the presence of ethanol (mock), 1 nM DHT, and/or 1 μM HF, as indicated. Luciferase activity analyzed in a luminometer is presented relative to that of mock treatment in each cell line (first lane; set as onefold). Each value represents the mean + S.D. from at least three independent experiments. *$P<0.001$ (versus mock treatment in 5637-AR). **$P<0.01$ (versus mock treatment in 5637-AR). ***$P<0.05$ (versus mock treatment in 5637-AR). #$P<0.001$ (versus DHT treatment in 5637-AR). (B) 5367-AR or 5637-V cells were cultured for 4 days in the presence of ethanol (mock), 1 nM DHT, 1 μM HF, and/or 50 nM PD168393 (PD), as indicated. Cell viability was assayed with MTT, and growth induction is presented relative to cell number with ethanol treatment estimated by measuring the absorbance at a wavelength of 570 nm with a background subtraction at 655 nm (first lane; set as onefold). Each value represents the mean + S.D. from at least three independent experiments. *$P<0.01$ (versus mock treatment in 5637-AR; lane 1). **$P<0.001$ (versus mock treatment in 5637-AR; lane 1). ***$P<0.01$ (versus mock treatment in 5637-V; lane 7). #$P<0.001$ (versus DHT treatment in 5637-AR; lane 2). ##$P<0.01$ (versus DHT treatment in 5637-V; lane 8).

DHT was decreased to 15% (lane 5 vs 6, $P=0.455$) (vs 60% increase without the inhibitor described above), suggesting that androgen effect on cell growth is at least partially mediated through the EGFR pathway. In 5637-V cells, PD168393 showed a lower rate (up to 54%) of growth suppression (Fig. 1B, lane 7 vs 11, $P=0.009$), and DHT did not stimulate the cell growth (lane 11 vs 12, $P=0.853$).

Androgen upregulates *EGFR* and *ERBB2* gene transcription

We assessed changes in *EGFR* and *ERBB2* mRNA levels following androgen treatment in AR-positive bladder cancer cells by real-time reverse transcription

(RT)-PCR. As shown in Fig. 2A, DHT treatment for 24 h increased EGFR levels to 1.7- and 1.9-fold in UMUC3 and TCC-SUP cell lines respectively, compared with mock treatment. Similarly, DHT treatment resulted in up to 1.7-fold increase in ERBB2 levels in both cell lines. As expected, HF blocked the effect of DHT on the expression of EGFR and ERBB2 in these cell lines.
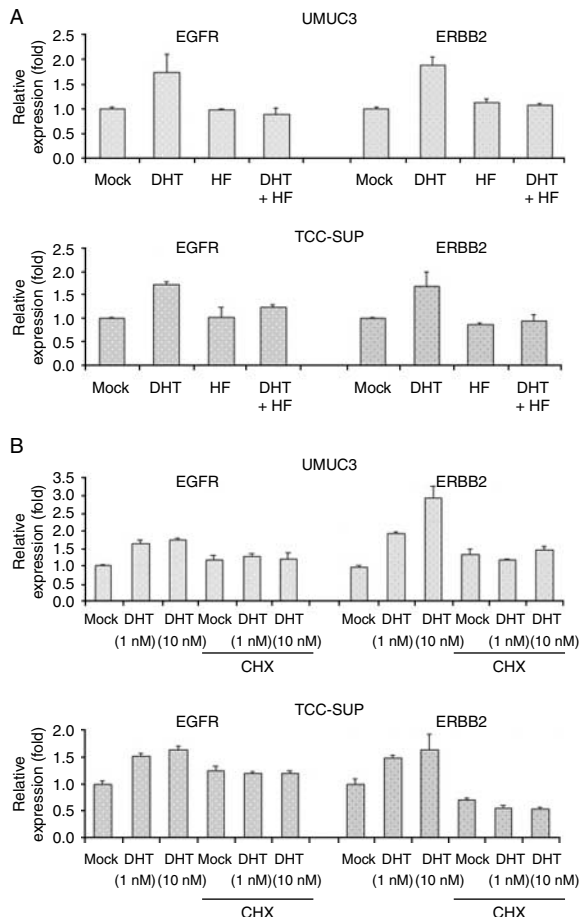

Figure 2 Regulation of *ERBB2* and *EGFR* gene transcription by androgen in bladder cancer cell lines. (A) RNA extraction and subsequent real-time RT-PCR for EGFR and ERBB2 were performed in UMUC3 and TCC-SUP cells treated with ethanol (mock), 1 nM DHT, and/or 1 μM HF for 24 h. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that of mock treatment in each cell line (first lane; set as onefold). Each value represents the mean+S.D. from at least three independent experiments. (B) UMUC3 or TCC-SUP cells cultured in the presence of ethanol (mock), 1 nM DHT, or 10 nM DHT for 24 h, with or without pretreatment of 25 μg/ml cycloheximide (CHX) for 30 min, were analyzed on real-time RT-PCR for EGFR and ERBB2. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that of mock treatment in each cell line (first lane; set as onefold). Each value represents the mean+S.D. from at least three independent experiments.

Ligand-activated AR is known to regulate the expression of its target genes either by serving as a transcription factor via directly binding to their regulatory sequences or by modulating other transcription factors (Nelson *et al*. 2002, Pignon *et al*. 2009). To validate the mechanism responsible for androgenic regulation of *ERBB2* and *EGFR* gene expression, we introduced cycloheximide prior to DHT treatment to block protein neosynthesis. As shown in Fig. 2B, both at 1 and 10 nM, DHT alone was found to increase EGFR and ERBB2 transcript abundance in both cell lines in a dose-dependent manner. In cycloheximide-treated cells, DHT lost its effect on influencing EGFR or ERBB2 transcription. These results suggest that androgen-mediated increase in *ERBB2* and *EGFR* mRNA levels requires novel protein synthesis and, therefore, androgens could indirectly stimulate gene expression of EGFR and ERBB2.

Androgen upregulates EGFR and ERBB2 protein expression

We also examined the effect of androgen on EGFR and ERBB2 expression in protein levels in UMUC3 and TCC-SUP cell lines. Cell extracts upon androgen/anti-androgen treatment were analyzed by western blot, using an antibody to EGFR or ERBB2. In accordance with their mRNA levels shown above, DHT increased EGFR and ERBB2 protein expression and HF antagonized the DHT effect (Fig. 3A). AR protein expression was similarly regulated by DHT and HF in these cells.

The AR not only functions as a transcription factor but also is shown to modulate the stability of protein (Perry & Tindall 1996). We again blocked the neosynthesis of protein, using cycloheximide pretreatment, and the degradation of EGFR and ERBB2 proteins at different time points in the presence or absence of DHT was determined in UMUC3 and TCC-SUP cells. As shown in Fig. 3B, there was no significant difference in the ratios of their protein degradation in the presence and absence of DHT. These findings indicate that androgen treatment has little influence on the stability of both EGFR and ERBB2 proteins in bladder cancer cells.

AR pathway is necessary for regulation of EGFR and ERBB2 expression by androgen

To further investigate whether upregulation of EGFR and ERBB2 expression by androgen is dependent on AR, stable bladder cancer cell lines (i.e. UMUC3-AR-shRNA versus UMUC3-control-shRNA and 5637-AR versus 5637-V) were analyzed by western blotting.
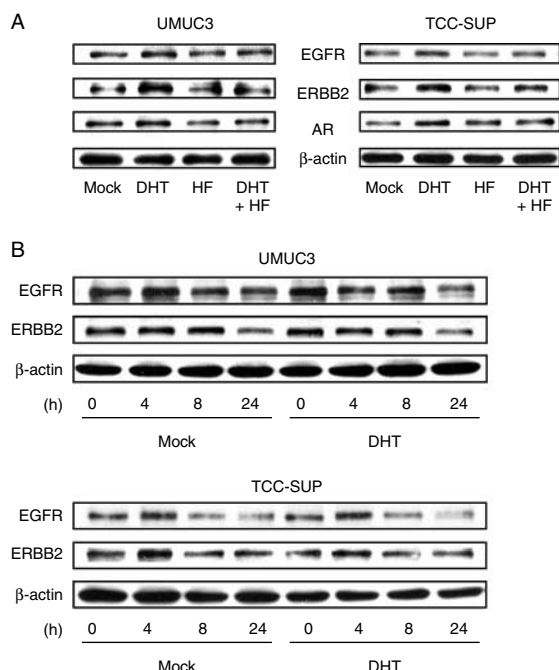
Figure 3 Regulation of ERBB2 and EGFR protein expression by androgen in bladder cancer cell lines. (A) UMUC3 or TCC-SUP cells were treated with ethanol (mock), 1 nM DHT, and/or 1 μM HF for 24 h. Equal amounts of protein extracted from each cell line were immunoblotted for EGFR (170 kDa), ERBB2 (185 kDa), or AR (110 kDa), as indicated. β-actin (43 kDa) served as the internal control. (B) UMUC3 or TCC-SUP cells were pretreated with 25 μg/ml cycloheximide for 30 min, and proteins were extracted at different time points after ethanol/1 nM DHT treatment for 0–24 h, as indicated. Western blots for EGFR, ERBB2, and β-actin were then performed.

We confirmed that the expression of AR-shRNA in AR-positive UMUC3 by retrovirus effectively silenced endogenous AR (Fig. 4A). Basal levels of EGFR and ERBB2 were much lower in UMUC3-AR-shRNA than in UMUC3-control-shRNA. AR-shRNA also abolished the effect of DHT on upregulation of EGFR and ERBB2. Interestingly, HF obviously increased EGFR and ERBB2 proteins via unknown pathway.

Protein levels of EGFR and ERBB2 were also compared in 5637-AR and 5637-V. Overexpression of AR lead to a dramatic increase in basal levels of EGFR, whereas it showed marginal effects on basal levels of ERBB2 (Fig. 4B). As expected, DHT increased EGFR and ERBB2 protein expression in 5637-AR, but not in 5637-V. EGFR and ERBB2 levels were marginally augmented by HF in AR-negative 5637-V cells.

Thus, it is likely that the AR is necessary for androgenic upregulation of EGFR and ERBB2 expression in bladder cancer cells.

Androgen promotes pEGFR and its downstream proteins

To examine whether AR activation influences the function of the EGFR pathway, we next investigated the phosphorylation status of EGFR and its downstream proteins AKT and ERK1/2 in AR-positive (UMUC3, 5637-AR) and AR-negative (5367-V) bladder cancer cell lines treated with DHT, EGF, and/or PD168393. In the presence or absence of EGF and PD168393, DHT increased the levels of EGFR in UMUC3 (Fig. 5A) and 5637-AR (Fig. 5B), but not in 5637-V (Fig. 5C). Furthermore, we detected eruption of pEGFR, pAKT, and pERK, but not EGFR, after introduction of EGF in all three bladder cancer cell lines, which was blocked by PD168393. However, additional increases in pEGFR, pAKT, and pERK were observed only in AR-positive UMUC-3 and 5637-AR cells after DHT treatment. In the presence of PD168393, DHT also slightly induced the levels of pAKT in UMUC3 cells, but not in 5637-AR and 5637-V cells. DHT and/or EGF did not affect the levels of AKT and ERK in these cells.

Immunoreactivity in bladder cancer tissue samples

We next performed immunohistochemical stains for AR, EGFR, pEGFR, ERBB2, pAKT, and pERK in 24
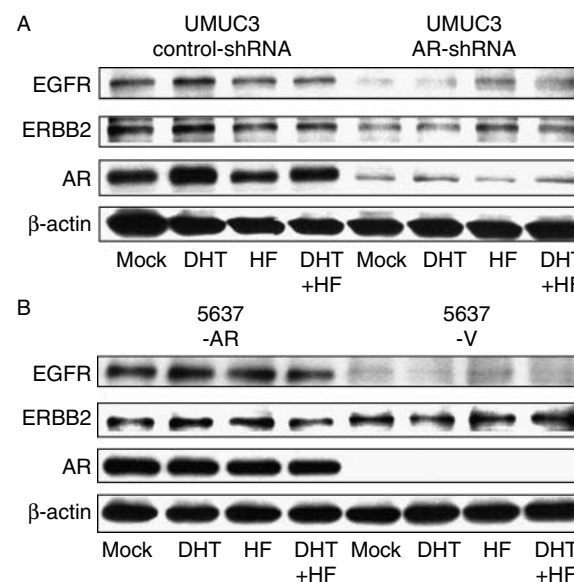
Figure 4 Effects of up/downregulation of AR on EGFR and ERBB2 expression in bladder cancer cells. UMUC3 stably expressing AR-shRNA or scrambled shRNA (A) or 5637 stably expressing AR or vector only (B) was treated with ethanol (mock), 1 nM DHT, and/or 1 μM HF for 24 h. Equal amounts of protein extracted from each stable cell line were immunoblotted for EGFR (170 kDa), ERBB2 (185 kDa), or AR (110 kDa), as indicated. β-actin (43 kDa) served as the internal control.
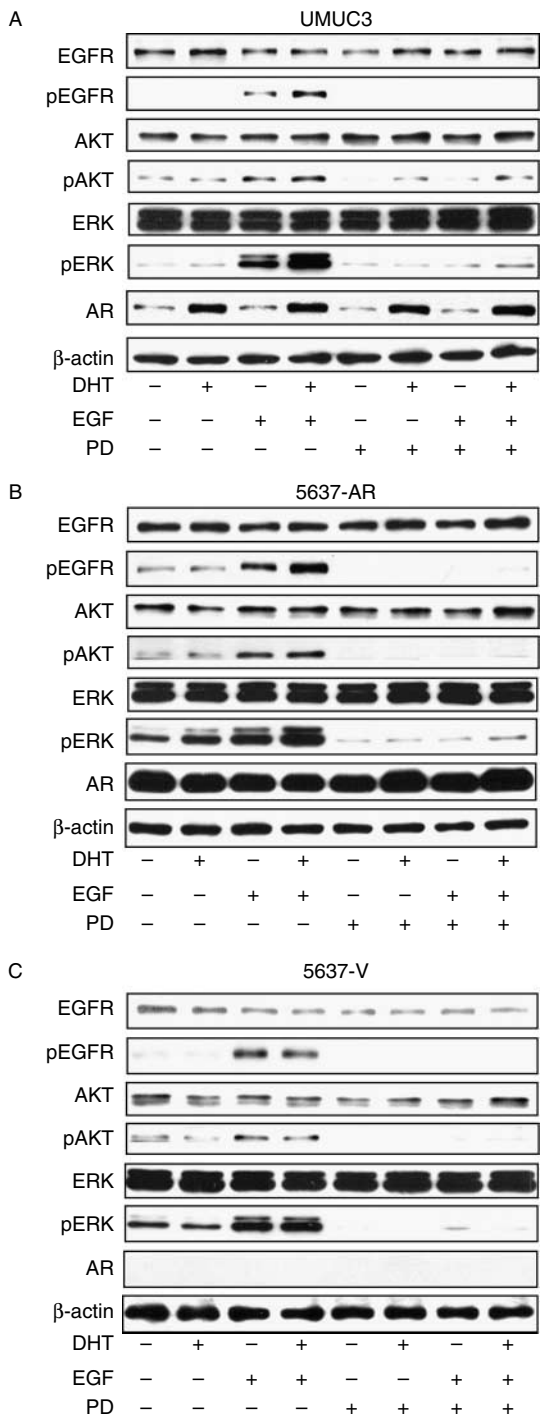
Figure 5 Phosphorylation of EGFR and its downstream proteins in bladder cancer cells. UMUC-3 (A), 5637-AR (B), or 5637-V (C) cells were pretreated with ethanol or 1 nM DHT for 24 h. Then, the cells were cultured with DMSO or 10 μM PD168393 (PD) for 45 min, followed by treatment with 20 ng/ml EGF for 15 min, as indicated. Equal amounts of protein extracted from each cell line were immunoblotted for EGFR, pEGFR, AKT, pAKT, pERK, ERK, AR, or β-actin (internal control), as indicated.

radical cystectomy specimens with high-grade urothelial carcinoma (Fig. 6). The results of the staining and correlations with pathological stage are summarized in Table 1. Overall, positive signals were found in 8 (33% for AR), 12 (50% for EGFR), 14 (58% for pEGFR), 17 (71% for ERBB2), 13 (54% for pAKT), and 13 (54% for pERK) cases. There were no statistically significant correlations between each staining and presence of muscle invasion ($\leq$T1 vs $\geq$T2) or lymph node metastasis, except an inverse correlation between pAKT positivity and tumor invasiveness ($P=0.0303$) and a positive correlation between pERK and node metastasis ($P=0.0498$). No significant association between gender of the patients and AR ($P=0.1028$), EGFR ($P=0.1584$), pEGFR ($P=0.0751$), ERBB2 ($P=0.4625$), pAKT ($P=0.1118$), or pERK ($P=0.1118$) was found. AR expression was then analyzed in comparison with respective stains: there were strong associations with EGFR ($P=0.0136$), pEGFR ($P=0.0041$), ERBB2 ($P=0.0331$), or pERK ($P=0.0274$), but not with pAKT ($P=0.5555$) (Table 1). Similarly, EGFR overexpression significantly correlated with the expression of pEGFR ($P=0.0005$), ERBB2 ($P=0.0343$), or pERK ($P=0.0498$), but not with that of pAKT ($P=0.5000$).

To assess possible associations between each expression and disease progression, we then performed the Kaplan–Meier analysis coupled with log-rank test. Of the 24 patients with a mean follow-up of 8.3 months, 8 (33%) developed recurrent/metastatic tumors after radical surgery. As shown in Fig. 7A, AR positivity was significantly associated with tumor progression ($P=0.0005$). Status of EGFR ($P=0.2425$; Fig. 7B), pEGFR ($P=0.1579$; Fig. 7C), ERBB2 ($P=0.2997$; Fig. 7D), and pERK ($P=0.1270$; Fig. 7F) tended to correlate with progression. Conversely, pAKT positivity was associated with significantly better prognosis ($P=0.0483$; Fig. 7E).

Discussion

There is increasing evidence to indicate that the AR, as a ligand-regulated transcription factor, significantly contributes to the development and progression of bladder cancer (Miyamoto *et al*. 2007, Johnson *et al*. 2008, Wu *et al*. 2010). Dysregulation of the EGFR/ERBB2 signaling pathway is well known to play an important role in the progression of bladder cancer (Neal *et al*. 1990, Lipponen & Eskelinen 1994, Orlando *et al*. 1996, Miyamoto *et al*. 2000, Jimenez *et al*. 2001, Bellmunt *et al*. 2003, Latif *et al*. 2004, MacLaine *et al*. 2008). However, the cross talk between AR and EGFR/ERBB2 pathways is uncharacterized in
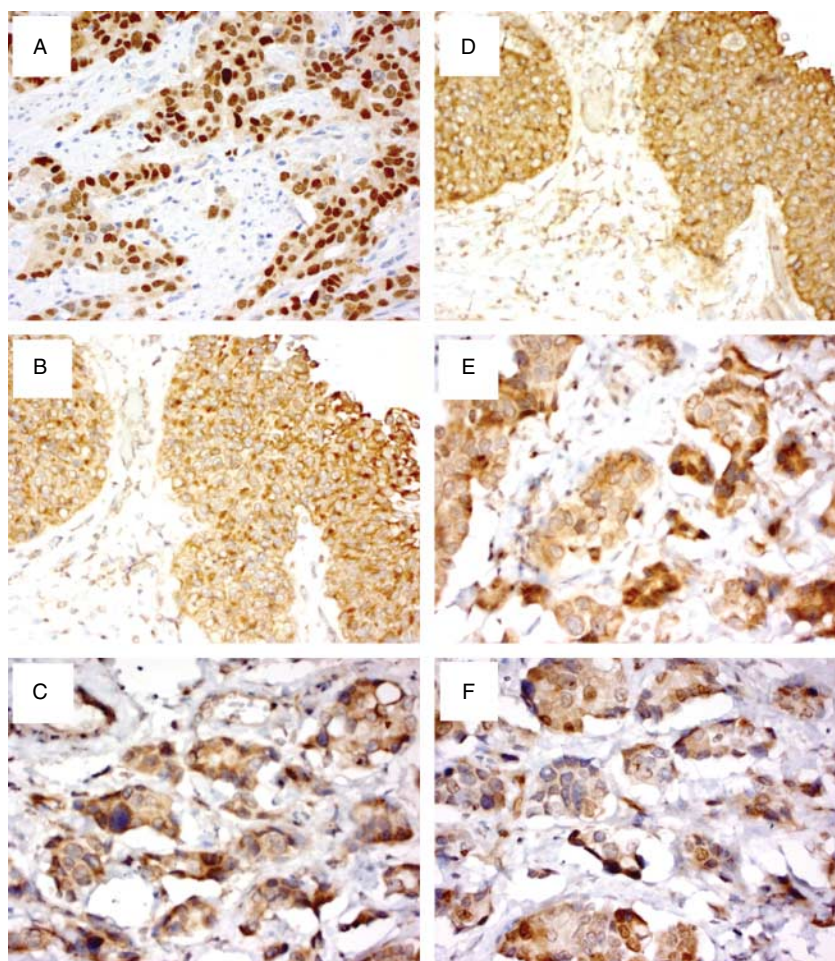
Figure 6 Immunohistochemistry of AR (A), EGFR (B), pEGFR (C), ERBB2 (D), pAKT (E), or pERK (F) in bladder cancer tissue samples. Original magnification, 400×.

bladder cancer, although it has been widely studied in prostate cancer (Culig *et al.* 1994, Scher *et al.* 1995, Craft *et al.* 1999, Signoretti *et al.* 2000, Di Lorenzo *et al.* 2002, Liu *et al.* 2005, Mukherjee & Mayer 2008, Pignon *et al.* 2009). In this study, we provide evidence that androgens regulate EGFR and ERBB2 expression via the AR pathway in bladder cancer cells.

In prostate cells, the AR likely has opposing effects on the regulation of EGFR expression: downregulation in benign cells and upregulation in malignant cells (Brass *et al.* 1995, Itoh *et al.* 1998, Traish & Morgentaler 2009). In prostate cancer cells, androgens were also reported to reduce EGFR protein expression (Mukherjee & Mayer 2008). The underlying mechanism responsible for the distinct effects needs to be determined. As shown for the regulation of AR activity by AKT in LNCaP prostate cancer line (Lin *et al.* 2003), such opposing effects might be dependent

on passage number of the cells in which AR status could be different (e.g. expression level and sensitivity to ligands). ERBB2 is generally repressed by androgens in prostate cancer cells (Berger *et al.* 2006, Pignon *et al.* 2009). In this study, we showed in bladder cancer cells that androgen could induce the expression of both EGFR and ERBB2. The upregulation was observed in two bladder cancer cell lines with endogenous AR and an additional cell line exogenously expressing the AR. We further confirmed the necessity of the AR pathway for the upregulation, using anti-androgen treatment and RNA interference strategy. In addition, using an EGFR inhibitor PD168393, we showed that androgen-induced growth stimulation of AR-positive bladder cancer cells could be mediated via the EGFR pathway. PD168393 is believed to be a selective inhibitor of EGFR tyrosine kinase activity (Fry *et al.* 1998). However, treatment with higher doses of PD168393 (e.g. 200 nM) resulted

Table 1 Expression of androgen receptor (AR), epidermal growth factor receptor (EGFR), phosphorylation of EGFR (pEGFR), ERBB2, pAKT, and pERK in 24 bladder cancer tissue microarrays

	Positive cases (%)	≤**T1** (*n*=5)	≥**T2** (*n*=19)	*P* value	**N(−)** (*n*=12)	**N(+)** (*n*=12)	*P* value	**AR(−)** (*n*=16)	**AR(+)** (*n*=8)	*P* value
AR	8 (33)	2 (40)	6 (32)	0.5547	2 (17)	6 (50)	0.0965	NA	NA	NA
EGFR	12 (50)	2 (40)	10 (53)	0.5000	5 (42)	7 (55)	0.3021	5 (31)	7 (88)	0.0136
pEGFR	14 (58)	4 (80)	10 (53)	0.2826	7 (55)	7 (55)	0.6549	6 (38)	8 (100)	0.0041
ERBB2	17 (71)	4 (80)	13 (63)	0.5375	8 (67)	9 (75)	0.5000	9 (56)	8 (100)	0.0331
pAKT	13 (54)	5 (100)	8 (42)	0.0303	8 (67)	5 (42)	0.2068	9 (56)	4 (50)	0.5555
pERK	13 (54)	2 (40)	11 (58)	0.4146	4 (33)	9 (75)	0.0498	6 (38)	7 (88)	0.0274

N, lymph node metastasis.

in similar rates of growth suppression between 5637-AR and 5637-V cells (data not shown) that exhibit distinct basal levels of EGFR (Fig. 4B), postulating the possibility of the inhibition of other pathways by this inhibitor.

It is worth noting that AR knockdown in UMUC3 and AR overexpression in 5637 resulted in significant decrease and increase respectively, in the basal levels of EGFR (both cell lines) and ERBB2 (UMUC3 only). These findings may raise the possibility of the constitutive activity of the AR. However, the presence of mutations in the endogenous *AR* gene, leading to its androgen-independent activation often found in prostate cancer, has not been described in bladder cancer cell lines. Furthermore, there were no significant differences in the basal levels of AR transcriptional activity and cell growth rate between UMUC3/TCC-SUP with and without AR knockdown (Miyamoto *et al.* 2007) and between 5637-AR and 5637-V (Fig. 1). The 5637 cell line exhibited a relatively high basal level of ERBB2 protein, and AR overexpression in this line may, therefore, increase ERBB2 expression only marginally in the absence of androgens (Fig. 4B). However, the precise reason for the difference in the effect of AR expression on ERBB2 levels between UMUC3 and 5637 cells remains uncertain.

Although several putative AREs in an EGFR promoter region were identified by a computer analysis, their functionality in reporter vectors has not been demonstrated (Quandt *et al.* 1995, Pignon *et al.* 2009). Chromatin immunoprecipitation assays showed that androgens induced the recruitment of RNA polymerase II to the EGFR proximal promoter in prostate cancer cells (Pignon *et al.* 2009), suggesting the stimulation of *EGFR* gene transcription by androgen-activated AR. However, it remains to be determined whether the AR directly binds to the promoter. In this study in bladder cancer cells, blocking of protein neosynthesis, using cycloheximide pretreatment, abolished androgen-mediated increase in

EGFR and ERBB2 transcripts, suggesting that androgens act through an androgen-dependent intermediary. Ligand-activated AR is known to have dual functions in regulating target proteins either by increasing transcription or by decreasing degradation. The regulation of EGFR expression by androgens was shown to be at the transcriptional level in prostate cancer cell lines (Traish & Morgentaler 2009). Our protein stability assays showing marginal effects of androgen suggest that the AR functions only as a transcription factor in regulation of both EGFR and ERBB2 in bladder cancer cells.

EGF stimulates tyrosine phosphorylation of its receptor by homodimerization of EGFR and activation of receptor tyrosine kinases (Chen *et al.* 1987). The Ras–Raf–MAPK and PI3K–AKT–GSK pathways are considered to be the main traditional downstream of EGFR (Anderson *et al.* 1990). In addition to increased expression/activity of EGFR, these downstream signaling cascades are frequently deregulated in neoplasms, leading to tumorigenesis and tumor progression (Bacus *et al.* 1996, Craven *et al.* 2003). We observed that androgen treatment for 24 h increased pEGFR levels, particularly with EGF treatment, only in the presence of functional AR. Similar increases in the levels of pAKT and pERK, but not in those of total AKT and ERK1/2, were observed in androgen-treated AR-positive bladder cancer cells. Although direct interactions between the membranous AR and the growth factor receptors are possible mechanisms of this cross talk, shorter durations of androgen treatment (e.g. 1 h) resulted in little induction in the expression or activation of the receptors (data not shown), suggesting the classic genomic effects of androgens on EGFR/ERBB2 in bladder cancer cells. Moreover, androgen treatment in the presence of the EGFR inhibitor still slightly increased the levels of pAKT expression in UMUC3 cell line, but not in 5637-AR or 5637-V cell line, postulating the involvement of non-EGFR pathway(s) in UMUC3 cells.
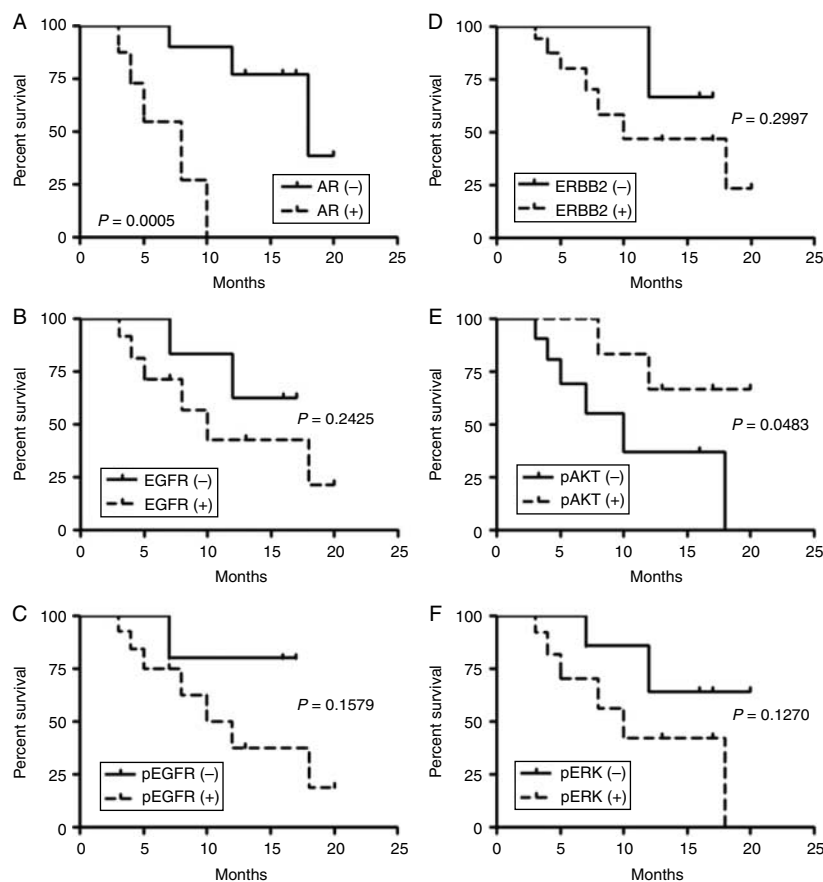
Figure 7 Progression-free survival rates in patients with bladder cancer. The Kaplan–Meier analysis was performed according to the expression of AR (A), EGFR (B), pEGFR (C), ERBB2 (D), pAKT (E), or pERK (F), and comparisons were made by log-rank test.

In prostate and breast cancers where androgenic regulation of the EGFR/ERBB2 pathways was shown, AR expression was found to significantly correlate with ERBB2 overexpression (Brys *et al*. 2004, Micello *et al*. 2010, Park *et al*. 2010). To investigate whether AR and EGFR/ERBB2 or phosphorylated forms of their downstream proteins co-express in bladder cancers, we immunohistochemically stained for AR, EGFR, pEGFR, ERBB2, pAKT, and pERK. As expected, there was a statistically significant association between EGFR overexpression and pEGFR expression as well as the status of ERBB2 or pERK. AR expression was then found to strongly correlate with the expression of EGFR, pEGFR, ERBB2, or pERK. These correlations strongly support the potential cross talk between AR and EGFR/ERBB2 pathways in bladder cancer.

Additionally, we analyzed the relationship between the immunostaining and tumor stage (i.e. degree of invasion and lymph node metastasis). In our cohort,

pAKT positivity showed an inverse association with deep invasion, confirming a recent report demonstrating significantly higher levels of pAKT in non-invasive bladder cancers than in invasive tumors (Schultz *et al*. 2011). However, earlier studies demonstrated similar overexpression of pAKT between non-invasive and invasive bladder tumors yet induction of invasive capacity of cancer cell lines via the PI3K/AKT pathway (Wu *et al*. 2004, Knowles *et al*. 2009). This study also analyzed and compared the prognostic value of respective expression, using Kaplan–Meier survival curves and log-rank test. Surprisingly, patients with AR-positive tumor had significantly higher risks of progression compared with those with AR-negative tumor. There are controversial data as to the correlation of AR expression in bladder cancer with tumor aggressiveness (Boorjian *et al*. 2004, 2009, Miyamoto *et al*. 2007, Mir *et al*. 2010, Tuygun *et al*. 2011). Few studies in which outcome differences among patients with muscle invasive disease were not analyzed

demonstrated a decrease in AR expression in higher grade/stage tumors (Boorjian *et al*. 2004, 2009, Tuygun *et al*. 2011). In contrast, a recent study analyzing 473 patients has revealed that AR positivity was significantly higher in muscle invasive tumors than in non-muscle invasive tumors (Mir *et al*. 2010). Nonetheless, in this study, there was no statistically significant difference in cancer-specific survival among patients with T2 disease. Our pilot study analyzing 33 superficial bladder carcinomas, using a quantitative RT-PCR method, showed that recurrence-free survival in patients with high AR-expressing tumors tended to be lower than that in patients with low AR-expressing tumors (Miyamoto *et al*. 2007). This study is the first to show a strong relationship between AR status and tumor progression. Because EGFR and ERBB2 were frequently overexpressed in aggressive bladder tumors (Neal *et al*. 1990, Lipponen & Eskelinen 1994, Orlando *et al*. 1996, Miyamoto *et al*. 2000, Jimenez *et al*. 2001, Latif *et al*. 2004) as discussed, we analyzed only high-grade, mostly invasive, urothelial carcinomas. The expression of EGFR, pEGFR, ERBB2, and pERK also tended to predict tumor progression. In addition to numerous previous studies on EGFR and ERBB2, a recent study has shown a possibility of pERK as a prognosticator in muscle invasive bladder tumors (Karlou *et al*. 2009). Indeed, there was a statistically significant association between pERK positivity and the presence of lymph node metastasis in our cohort. We further found that higher pAKT expression in bladder cancer predicted a better prognosis. However, this finding was inconsistent with not only our current data showing an increase in pAKT expression by androgen in AR-positive bladder cancer cells but also published evidence suggesting tumor progression via activation of PI3K/AKT signals (Wu *et al*. 2004, Knowles *et al*. 2009). Underlying mechanisms responsible for the protective roles of AKT activation in bladder cancer progression remain unclear. Therefore, further studies including larger patient cohorts with longer follow-up are needed to validate these initial results.

In conclusion, our study demonstrates that androgen upregulates the levels of EGFR and ERBB2 expression, as well as the activity of EGFR signaling, through the AR pathway in bladder cancer cells. Thus, AR signals may play an important role in the regulation of the EGFR/ERBB2 pathways, leading to the progression of bladder cancer. These results may significantly enhance previous findings suggesting the involvement of the AR pathway in bladder cancer and the consequence of androgen deprivation as a potential therapeutic approach.

Declaration of interest

Funding

Author contribution statement

Y Zheng and K Izumi performed the experiments and data analysis. J L Yao provided bladder cancer TMA and evaluated patient information. H Miyamoto conceived of the study, coordinated and supervised the project, and also evaluated immunohistochemical staining. Y Zheng drafted the manuscript and H Miyamoto edited it.

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The Role of the Androgen Receptor in the Development and Progression of Bladder Cancer

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Men are at a higher risk of developing bladder cancer than women. Since bladder cancer cell lines and tissues were found to express the androgen receptor, efforts have been made to inspect whether androgen-mediated androgen receptor signals are implicated in bladder carcinogenesis as well as cancer progression. Mounting evidence supports the view that bladder cancer is a member of the endocrine-related tumors and may clearly explain the gender-specific difference in the incidence. However, the underlying mechanisms of how androgen receptor signals regulate bladder cancer growth are still far from fully characterized. Moreover, it remains controversial whether the androgen receptor pathway always plays a dominant role in bladder cancer progression. In this review, we summarize the available data on the involvement of androgen receptor signaling in bladder cancer. In particular, current evidence demonstrating the stimulatory effects of androgens on tumor progression or, more convincingly, tumorigenesis via the androgen receptor pathway may offer great potential for androgen deprivation as a therapeutic or chemopreventive option in patients with bladder cancer.

Key words: androgens — androgen receptor — bladder cancer — carcinogenesis — progression

INTRODUCTION

Bladder cancer, mostly urothelial carcinoma, is the second most common genitourinary malignancy, leading to significant morbidity and mortality (1–4). Unlike most epithelial tumors, divergent pathways of tumorigenesis are involved in urothelial carcinoma (2–5). These separate mechanisms result in different biological behaviors and phenotypic variants, which gives rise to at least two distinct clinicopathologic types of neoplasms: non-invasive low-grade tumor and high-grade, often invasive, carcinoma. Patients with low-grade tumor generally have a favorable prognosis after transurethral tumor resection with or without intravesical pharmacotherapy, but they carry a lifelong risk of frequent recurrence (50–70%) with occasional progression to invasion. Those with the other, high-grade muscle-invasive tumor, even when given radical cystectomy with or without

systemic chemotherapy, are at a high risk for metastasis. Interestingly, owing to the lifelong need for monitoring for recurrence, the typical cost incurred by a bladder tumor patient from diagnosis to death has been reported to be the highest among all cancers (6). In addition, from 1990 to 2006, despite improvements in surgical technique and perioperative care, compared with prostate cancer (39% decrease), bladder cancer mortality was decreased only by 5% (3,7). Thus, identification of the molecules playing a key role in bladder cancer development and progression is urgently needed to improve the diagnosis, treatment and monitoring of the patients.

Comparative studies have demonstrated that men are three to four times more likely to develop bladder cancer than women, while female patients present with more aggressive tumors than male patients (3,4,8). In the USA, there will be

projected deaths of 10 510 men and 4370 women from bladder cancer in 2012, accounting for 18.9 and 24.4% of newly diagnosed cases, respectively (4). Environmental or lifestyle factors, such as industrial chemicals and cigarette smoke, have been blamed for the gender-specific differences in bladder cancer incidence and aggressiveness. However, even after controlling these carcinogenic factors, bladder cancer remains a predominant disease among men (4,9,10). Thus, the exact mechanisms responsible for the gender differences remain unclear. Recent studies have provided data supporting the hypothesis that androgen receptor (AR) signaling plays an essential role in the development and progression of bladder cancer, which may explain some of the differences between male and female tumors.

In this article, we review molecular evidence suggesting the modulation of bladder tumorigenesis and tumor progression through the AR pathway. We also highlight alterations of genetic pathways regulated by androgens in bladder cancer cells, which might contribute to development of prognostic biomarkers and/or novel targeted therapies.

ANDROGEN RECEPTOR

The AR, located on the X chromosome (q11-12), is well-known as a ligand-inducible transcription factor that regulates target gene expression (11−13). As a member of the nuclear receptor superfamily, the *AR* gene consists of eight exons that encode four structurally and functionally distinct domains: the NH_2-terminal transactivation domain, the DNA-binding domain, a hinge region and the COOH-terminal ligand-binding domain (11). The AR mediates its physiological activities by binding to androgens. Testosterone, upon entry into the target cell, binds to the AR directly or after conversion to 5α-dihydrotestosterone (DHT) by 5α-reductases. Non-ligand-bound AR is usually located in the cytoplasm where it associates with heat shock proteins (HSPs). Alternatively, this transcriptionally quiescent AR can be degraded via E3 ubiquitin ligase. The ligand-AR complex induces a conformational change in the AR, resulting in release of the HSPs and translocation of the complex to the nucleus. Sequentially, in a homodimeric fashion, the activated AR binds to the tissue-specific androgen-response element and recruits further proteins, such as general transcription factors and RNA polymerase II, leading to specific transcriptional activation or repression of target genes. In addition, primarily in prostate cancer cells, ligand-independent activation of the AR pathway by, for instance, peptide growth factors such as epidermal growth factor (EGF), has been demonstrated, presumably through signal transduction pathways (12).

Ligand-mediated receptor transactivation can be further modulated by a number of co-regulatory proteins, termed coactivators and corepressors (13). It has been well acknowledged, especially in prostate cancer cells, that the transcriptional activity of AR is dependent on AR-coregulator complex composition. Increased affinity between AR and coregulator is generally associated with ligand binding, which subsequently enhances AR transactivation by facilitating DNA occupancy, chromatin remodeling, ensuring AR protein stability and proper AR subcellular distribution (11−13).

DISTRIBUTION OF THE AR AND THE PHYSIOLOGICAL FUNCTION OF ANDROGENS IN THE BLADDER

The AR is ubiquitously distributed throughout the human body despite gender difference and even in mouse, rat and monkey tissues (14,15). Indeed, a wide range of biological actions of androgens, such as maintaining libido, spermatogenesis, muscle mass and strength, bone mineral density and stimulating erythropoiesis, are known (16). AR expression has also been detected widely in the bladder, including urothelium, muscularis propria (detrusor muscle) and neurons (14,15,17−19).

Increasing evidence from animal and human studies has shown that androgens contribute to urinary tract functions. In studies in male animals, androgens inhibited bladder detrusor muscle contraction via neuronal regulation (20−22). In castrated rat bladder, androgen replacement augmented urothelial thickness, muscle fiber quantity and vessel number (23). Androgen deprivation dramatically down-regulated the activity and expression of tissue enzymes involving cholinergic and non-cholinergic nerve functions (24,25). In a recent study, castration in male rats, via transforming growth factor-β, led to decreases in maximal volume and compliance of the bladder, and androgen supplementation restored bladder dysfunction (26). These findings in animal models suggested that androgens might directly regulate voiding function. In humans, it was suggested that there is a correlation between androgen deficiency and bladder dysfunction (27). A few other studies have shown improvement of lower urinary tract symptoms in men treated with testosterone (28,29).

AR ALTERATIONS IN BLADDER CANCER

Intense efforts have been made to examine the expression of the AR in bladder cell lines and tissue specimens. Table 1 summarizes the results from such studies in human tissue samples and the correlation of AR expression with the clinicopathologic profile of the patients.

Several human bladder cancer cell lines have been found to express the AR at messenger RNA (mRNA) and protein levels (30−34). Using androgen-binding assays in relatively small numbers of tissue specimens, the presence of the AR in bladder cancer was suggested (35,36), although another study failed to show positivity in all the tissues examined (13 bladder and 3 ureter tumors) (37). Subsequently, immunohistochemical analyses have demonstrated that 44−78% of

Table 1. Androgen receptor (AR) expression in bladder cancer and its correlation with clinicopathologic features

Years (reference)	Method	n	AR expression			Gender			Tumor grade			Tumor stage			Prognostic significance
			Non-tumor	Tumor	P value	M	F	P value	L	H	P value	NMI	MI	P value	
1985 (35)	Binding assay	13	17.2 (Fm/mg)	49.5 (Fm/mg)	NA	68 (Fm/mg)	27.7 (Fm/mg)	NA	M: 43.8, F: 27.7 (Fm/mg)	32.4 (Fm/mg)	NA	NA	NA	NA	NA
1986 (36)	Binding assay	6	NA	83%	NA	100%	75%	1.000*	G2: 67%	G3–4: 100%	NA	Ta: 67%	Met: 100%	NA	NA
1990 (37)	Binding assay	13	NA	0%	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1997 (38)	IHC	9	NA	78%	NA	100%	33%	0.083*	NA	G2: 50%, G3: 100%	NA	T1: 80%	T3: 75%	1.000*	NA
2004 (39)	IHC	17	0%	52%	NA	NA	NA	NA	NA	G3: 52%	NA	NA	NA	NA	NA
2004 (40)	IHC	49	86%	53%	0.001*	61%	30%	0.104*	89%	49%	0.055*	75%	21%	0.002	NA
			M: 82%	M: 61%	0.101*										
			F: 100%	F: 30%	0.002*										
2007 (31)	RT–PCR	33	NA	100%	NA	NA	NA	NA	NA	NA	NS	100%	NA	NS (Ta vs. T1)	Rec: high(+) = higher risk (P < 0.1)
2009 (33)	IHC	55	NA	44%	0.06	NA	NA	NA	NA	NA	NA	59%	33%	0.095*	NA
2011 (41)	IHC	139	0%	M: 53%	<0.001*	53%	43%	0.481*	64%	37%	0.002*	60%	21%	<0.001*	Rec: (+) = lower risk (P = 0.095); Prog: (+) = lower risk (P = 0.110)
2011 (42)	IHC	472	NA	13%	NA	14%	8%	0.159*	12%	13%	0.864*	9%	15%	0.063*	NS
2011 (45)	IHC	93 (UUT)	NA	12%**	NA	NA	NA	NA	NA	NA	0.074*** (L>H)	NA	NA	0.001 (Stage II > I)	NS (P = 0.568***)
2011 (34)	IHC	59	84%	Roughly half	<0.001	NA	NA	0.961	NA	NA	NA	NA	NA	0.028 (NMI > MI)	NS
2012 (43)	IHC	188	U: 80%, S: 50%	42%	<0.001 (U), 0.181 (S)	42%	43%	1.000	55%	36%	0.023	51%	33%	0.018	NS (Prog in MI: (+) = higher risk, P = 0.071)

M, male; F, female; IHC, immunohistochemistry; RT-PCR, reverse transcription-polymerase chain reaction; NA, not assessed or not available; NS, not significant; L, low-grade; H, high-grade; NMI, non-muscle-invasive; MI, muscle-invasive; Met, metastasis; Rec, recurrence; Prog, progression; UUT, upper urinary tract tumor; U, benign urothelium; S, benign stroma.
*We calculated the two-tailed P value using Fisher's exact test.
**High (>10% of cells stained) nuclear expression.
***High (>10% cells stained) vs. low cytoplasmic expression.

bladder tumors express the AR (33,34,38−41). In contrast, in a recent and the largest study involving 492 patients, only 13% of bladder tumors were found to show AR expression (42). Our most recent study (43) showed that AR stained positively in 79 (42%) of 188 bladder tumors. Of note, there was thus a discrepancy in AR positivity between the two studies [i.e. 13% (42) *vs.* 42% (43)] where we stained the bladder tissue microarrays (TMAs) constructed at different institutions, using the same antibody and protocol. As shown in the estrogen receptor expression in breast cancer (44), the type and the duration of tissue fixation may have dramatically altered the levels of immunoreactivity to AR. Indeed, in our previous pilot study, using a quantitative reverse transcription−polymerase chain reaction method, AR signals were detected in all the mRNAs isolated from 33 fresh bladder tumors (31).

In some of the previous studies where control tissues were also assessed (34,40,43), higher rates of AR positivity were observed in benign bladders than in tumors. Conversely, few other studies failed to show higher expression of AR in benign bladder specimens, compared with tumors (33,35,39,41). To date, no studies have shown a statistically significant difference in AR expression between tumors from men and women (34,36,38,40−43). Interestingly, in a study (40), a statistically significant decrease in AR levels was seen in female tumors, but not in male tumors. Most of the studies demonstrated a significant decrease in AR expression in higher grade and/or stage tumors (33,34,40,41,43,45). However, in the largest study (42), there was no significant difference between low-grade (12%) and high-grade (13%) tumors. In addition, in other studies, no significant difference in AR expression between different stages of tumors (38) or even statistically significant higher expression levels in more advanced tumors (42,45) were observed. Moreover, no statistical significance in AR positivity as a prognosticator has been reported. We showed that AR positivity tended to correlate with recurrence in superficial tumors (31) or progression in muscle-invasive tumors (43), while others either failed to show any tendency (34,42,45) or showed an opposite tendency (41).

Thus, the available data obtained by immunohistochemistry regarding the correlation of AR expression in bladder cancer with tumor characteristics remain controversial. This may result from different methods of tissue preparation (e.g. preservation in fixative, embedding in paraffin, TMA construction and sectioning) and staining (e.g. antibody, protocol and criteria for positivity). Nonetheless, predominant results have suggested significant decreases in AR expression in bladder cancer compared with benign urothelium and in high-grade/invasive tumors compared with low-grade/superficial tumors. Further studies are warranted to determine the actual frequency of AR expression and its significance in differences in tumor aggressiveness (e.g. tumor grade, stage, size and multiplicity) and patients' outcome as well as other factors of the patients such as age, gender and history of smoking that is known as a risk factor.

In our recent immunohistochemical study (43) described earlier, benign stromal cells in approximately half of the bladder cancer cases were also found to express the AR. Indeed, stromal AR in the prostate has been shown to play a key role in its carcinogenesis and cancer progression (46). In bladder cancer, several proteins originated from stromal cells as well as tumor−stroma interactions are known to contribute to its growth (47,48). However, to our knowledge, no attempt has been made to elucidate the role of stromal AR in bladder cancer.

In addition to differential AR expression, genetic alterations involving *AR* gene have been described in bladder cancer. Allelic loss of the AR locus was identified in all the three informative cases of female muscle-invasive tumors, but not in corresponding non-neoplastic tissues from the same section of cystectomy (49). A recent study involving 95 male patients with bladder cancer demonstrated a significantly shorter CAG (glutamine) repeat length in exon 1 of the *AR* gene (mean: 20.0), predictive of higher transactivation activity, compared with 95 control males (mean: 21.1) (50). In an earlier study (51), men and women who had 23 and 44 (cumulative) CAG repeats, respectively, were also found to have a significantly elevated risk of urothelial carcinoma, compared with those with longer CAG. The sequencing of mRNAs from two human bladder cancer cell lines revealed a wild-type AR sequence with short CAG repeat lengths (20 in UMUC3 and 22 in TCC-SUP) (33). All these findings suggest that *AR* gene alterations are involved in bladder tumorigenesis.

AR COREGULATORS AND BLADDER CANCER

As described, androgen-mediated AR transcription can be further activated by coactivators. In prostate cancer, up-regulation of various AR coactivators has been observed during tumorigenesis and cancer progression, and deregulated expression of many of these coactivators has been shown to correlate with poor prognosis (12,13,52). AR coregulators have also been investigated in bladder cancer cell lines and tissue samples. First, overexpression of a general steroid hormone receptor coactivator AIB1/SRC-3 was detected in approximately one-third of bladder cancer, which significantly correlated with higher grade/stage and poorer prognosis (53). Second, the expression of AR coactivators, including NCOA1, NCOA2, NCOA3, CREBBP and EP300, was detected in AR-positive bladder cancer cell lines as well as 44−100% of bladder cancer tissue samples even in some of which AR was lacking (33). Among these five coactivators, only NCOA1 showed a significant decrease in its expression levels in tumors, compared with non-neoplastic urothelium. Nonetheless, small interfering RNA (siRNA)-mediated knockdown of any of the coactivators led to marked decreases in androgen-induced proliferation of bladder cancer cells. Remarkably, expression levels of these AR coactivators in bladder cancer cells were not correlated

with AR status nor affected by androgen treatment, suggesting alternative mechanisms of coregulator functions in urothelial carcinoma *vs.* in other AR-positive malignancies such as prostate cancer. Finally, the expression of JMJD2A and LSD1 that mediate AR transcription via histone lysine-demethylation mechanisms was significantly reduced in muscle-invasive bladder cancer, although JMJD2A and LSD1 levels were significantly lower and higher, respectively, in malignant versus benign urothelium (34). Loss of JMJD2A was correlated with worse overall survival ($P = 0.033$), but not with disease-free ($P = 0.409$) or cancer-specific ($P = 0.761$) survival. Furthermore, LSD1 inhibitors suppressed cell proliferation and androgen-induced expression of the AR-regulated neutral endopeptidase (*NEP*) gene in AR-positive bladder cancer lines. Thus, the current data may not only help in identifying AR coregulators as novel prognostic markers but also suggest the involvement of the AR-coregulator complex in bladder carcinogenesis and cancer progression.

AR SIGNALING IN BLADDER TUMORIGENESIS

Because the urothelium is primarily derived from the urogenital sinus during embryogenesis, which in males also gives rise to the prostate, similar mechanisms of AR regulation may exist in the bladder and prostate. The available data on AR alterations between malignant and benign urothelium also support the involvement of AR signals during the development of bladder cancer.

N-Butyl-N-(4-Hydroxybutyl) Nitrosamine in Animal Models

Industrial chemicals such as aromatic amines, compounds found in tobacco and tobacco smoke such as 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and arsenic are well-known bladder carcinogens (54,55). In experimental rodents, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), which can efficiently induce bladder tumors from urothelial dysplasia and carcinoma *in situ* to invasive carcinoma (54), has been commonly used as a suitable model for bladder tumorigenesis.

Using BBN and animal models, others and we have assessed the role of androgens and/or AR signals in bladder carcinogenesis (31,56−59). Consistent with epidemiological findings of male dominance in human bladder cancer (3,4), BBN was shown to more frequently and/or more rapidly induce bladder tumors in male animals than females (31,56,57). In male animals, surgical (bilateral orchiectomy) or medical (diethylstilbestrol or luteinizing hormone-releasing hormone agonist injection) castration, as well as administration of an AR antagonist flutamide, reduces or retards the occurrence of BBN-induced bladder cancer

(31,57,58). However, a 5α-reductase inhibitor finasteride showed marginal inhibitory effects on bladder tumor development (58), suggesting that testosterone itself may be a potent promoter of bladder carcinogenesis. Unilateral orchiectomy also led to a significantly higher incidence of bladder cancer, possibly because of a transient increase in the level of testosterone produced from the contralateral testis (59). In female animals, administration of testosterone with or without castration (bilateral ovariectomy) increased the bladder tumor incidence (57). Using an AR-knockout (ARKO) mouse model, we further showed that lack of a functional AR completely prevented bladder cancer development (31), suggesting that AR signals are essential for promoting bladder carcinogenesis. Unexpectedly, a subset of BBN-treated ARKO males supplemented with DHT eventually developed bladder cancer (31). Thus, our results showing the differences in cancer incidence at 40 weeks between castrated males (50%) and ARKO males (0%) and between DHT-supplemented ARKO males (25%) and ARKO males/females (0%) suggested the involvement of non-androgen-mediated AR pathways and androgen-mediated non-AR pathways, respectively, in inducing bladder carcinogenesis.

Other Animal Models

Using mouse xenograft models, the role of AR signals in bladder carcinogenesis was further investigated. Tumor development of the R198 transplantable line, derived from a human bladder carcinoma expressing the AR, was prevented by castration in 50% of adult male mice (60). Targeting the AR via the expression of AR-siRNA in xenograft tumor cells or administration of anti-AR molecule ASC-J9 that selectively degrades AR protein (61) in mice also resulted in delayed tumor formation (31).

Effects of androgens and AR signals on the expression of enzymes that activate or inactivate carcinogens have also been assessed in non-neoplastic bladder cells. Castration down-regulated cytochrome P450 CYP4B1, which activates amines to more genotoxic substances, in male rat bladders, and testosterone supplement restored CYP4B1 levels (62). We have recently shown that AR signals reduce the levels of UDP-glucuronosyltransferase [UGT (for human)/Ugt (for mouse)], which is known to play an important role in detoxifying bladder carcinogens, such as BBN and NNAL (55). In the SVHUC human normal urothelial cell line stably expressed with AR, DHT treatment down-regulated the expression of UGT subtypes, and flutamide antagonized the DHT effects. Additionally, Ugt levels were higher in mouse bladders from wild-type females than those from wild-type males, those from castrated males than those from intact males and in those from ARKO males than those from wild-type littermates. The findings from these two studies (55,62) suggest that androgen-mediated AR signals promote bladder carcinogenesis by up-regulating CYP4B1 and down-regulating UGTs in the bladder.

AR SIGNALING IN BLADDER TUMOR PROGRESSION

Although it remains controversial whether the AR pathway always plays a dominant role in bladder cancer progression, evidencing the effects of androgens on the growth of AR-positive tumors *in vitro* and *in vivo* are promising.

IN VITRO EFFECTS OF ANDROGENS-AR

Several *in vitro* analyses have assessed the effects of androgens and/or AR signals on the growth of bladder cancer cells. First, AR-mediated transactivation can be modulated by androgens in bladder cancer cells (30,31,63). Androgens increased AR-responsive reporter gene activity, which was abolished by AR antagonists or AR knockdown via RNA interference technology, indicating the functional activity of their endogenous AR. Second, AR expression can be altered by androgen treatment in bladder cancer cells. Boorjian et al. (33) showed that treatment with 1 nM R1881, a synthetic androgen, for 48 h resulted in a considerable decrease/little change in AR protein expression in UMUC3/TCC-SUP cells, respectively. Our western blot analysis then showed modest increases in endogenous AR expression in both lines, but not in exogenously overexpressed AR in AR-negative 5637 cells, after DHT treatment at 1 nM for 24 h (63). Third, AR signals have been shown to have stimulatory effects on bladder cancer cell growth (31–33,63,64). In cell viability assays, androgens induced AR-positive cell proliferation, and anti-AR compounds (e.g. flutamide, ASC-J9) or silencing of AR eliminated the effect of androgens. AR knockdown in bladder cancer cells was also shown to result in increased apoptosis and decreased migration in the presence or absence of androgens (64). Finally, as shown in prostate cancer cells, EGF, in conjunction with androgen, augmented AR transcriptional activity and protein expression as well as cell proliferation in bladder cancer lines, suggesting the EGF effects through activation of the AR pathway (65). Taken together, *in vitro* evidence strongly supports the stimulatory role of AR signaling in bladder cancer progression.

IN VIVO EFFECTS OF ANDROGENS-AR

In an earlier study using R198-bearing male mice, the tumors grew more rapidly following administration of DHT than those in untreated controls (60). Subsequently, using mouse xenograft models for AR-positive bladder cancer, others and we have shown that androgen deprivation via castration and/or flutamide or ASC-J9 treatment (31) as well as AR silencing via electroporation to deliver AR-siRNA (64) significantly reduces tumor size. Coincidentally, decreased cell proliferation index and increased apoptotic index, as well as decreases in the expression of angiogenesis/metastasis-related factors, including basic fibroblast growth factor, vascular endothelial growth factor and matrix metalloproteinase (MMP)-9, were observed in these xenograft tumors where the AR was targeted (31,64). Inhibitory effects of androgen ablation on tumor progression were confirmed further in the UPII-SV40T transgenic mouse model that expresses the SV40 large T antigen particularly in the urothelium and spontaneously develops bladder cancer (32). Castration retarded tumor growth and increased the expression of thrombospondin-1 (TSP1), which inhibits angiogenesis, while DHT supplement restored the effects of androgen ablation on tumor size and TSP1. Thus, *in vivo* studies have attempted to determine the clinical relevance of *in vitro* findings and to address the feasibility of future therapeutic application.

Most recently, anti-tumor effects of the bladder-cancer-specific adenovirus carrying E1A-AR were assessed both *in vitro* and *in vivo* (66). Infection of the viruses targeting AR-positive bladder cancer led to an inhibition of cell proliferation and a regression of implanted tumors.

AR-REGULATED MOLECULES IN BLADDER CANCER CELLS

Through the aforementioned *in vitro* studies, it has been shown that androgens are able to modulate the expression/activity of various molecules via the AR pathway in bladder cancer cells. These molecules related to cell proliferation, tumor growth and/or metastasis are summarized in Table 2.

Activation of the EGF receptor (EGFR) family, such as EGFR and ERBB2, is known to involve tumorigenesis and tumor progression of a variety of malignancies, including bladder cancer. Consequently, the efficacy of targeted

Table 2. Reported effects of androgens/AR signals on the expression/activity of key molecules related to tumor growth in bladder cancer cells

Molecules	Main function	Androgen effect	Reference
MMP-9	Cell proliferation, migration and invasion	Up-regulation	64
Caspase-3/7	Apoptosis executor	Down-regulation	64
Cyclin D1	Cell cycle regulator	Up-regulation	64
Bcl-xL	Anti-apoptotic factor	Up-regulation	64
NEP	Cell surface metalloprotease	Up-regulation	34
EGFR	Cell proliferation, migration and invasion	Up-regulation	63
ERBB2	Cell proliferation, migration and invasion	Up-regulation	63
AKT	Cell survival	Up-regulation	63
ERK1/2	Cell survival	Up-regulation	63
IL-6	Cytokine	Down-regulation	30
β-Catenin	Cell growth and adhesion	UP-regulation	69

MMP, matrix metalloproteinase; NEP, neutral endopeptidase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IL, interleukin.

therapy directed at EGFR signals has been assessed in bladder cancer (67). Recently, we demonstrated that androgen up-regulated the expression of EGFR and ERBB2 as well as the levels of phosphorylation of their downstream proteins AKT and extracellular signal-regulated kinase1/2 via the AR pathway in bladder cancer cells (63). Together with our other recent findings showing EGF-induced cell proliferation via modulating AR signals (65), cross-talk between AR and EGFR pathways was suggested to play an important role in bladder cancer progression.

Intravesical administration of bacillus Calmette-Guerin (BCG) is so far the most effective form of adjuvant therapy for high-risk superficial bladder cancer. Among cytokines elicited in response to BCG, interleukin (IL)-6 likely contributes to promotion of BCG adherence to bladder cancer cells and consequently to determination of BCG treatment efficacy. In AR-positive bladder cancer lines, DHT down-regulated BCG-induced IL-6 expression, and antiandrogens reversed the DHT effects (30). These results suggest that pharmacological manipulation of AR-mediated suppression of IL-6 has therapeutic value during intravesical BCG treatment.

Activation of Wnt/β-catenin signaling has been reported to correlate with poor prognosis in patients with bladder cancer (68). In our bladder TMAs, co-expression of nuclear AR and β-catenin was associated with tumor progression. In AR-positive bladder cancer lines, we further showed that DHT increased the expression of an active form of β-catenin and enhanced its nuclear translocation (69). Thus, it appeared that androgen was able to activate β-catenin signaling via the AR pathway in bladder cancer cells.

CONCLUSIONS

Given the current understanding of a critical role for AR signaling, as well as the involvement of other nuclear hormone receptor signals (70), in bladder tumorigenesis and tumor progression, bladder cancer should be accepted as a member of endocrine-related neoplasms. Although the underlying mechanisms of how AR signals regulate bladder cancer growth remain far from fully understood, the available data strongly support that targeting the AR provides effective chemopreventive and therapeutic approaches for urothelial carcinoma. Indeed, a variety of therapeutic options are available for AR-dependent prostate cancer (12), and most of these anti-AR therapies may be able to be applied to bladder cancer. Their preventive roles appear to be more convincing because the AR pathway is likely essential for bladder cancer initiation, while there are no clinical data showing that androgen deprivation therapy in prostate cancer patients reduces the incidence of subsequent bladder cancer. In contrast, the usefulness of anti-AR therapy may be limited, for example, for patients with bladder cancer possessing a functionally active AR. Further understanding of the roles of AR as well as other molecules directly or indirectly regulated by androgens may help to develop better strategies for the management of bladder cancer.

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Conflict of interest statement

None declared.

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Original contribution

Seminal plasma proteins in prostatic carcinoma: increased nuclear semenogelin I expression is a predictor of biochemical recurrence after radical prostatectomy ☆

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Summary Semenogelins and eppin are seminal plasma proteins that form a complex and inhibit sperm motility. However, the role of these proteins in prostate cancer is poorly understood. We immunohistochemically stained for semenogelins I and II and eppin in 291 radical prostatectomy specimens. We then evaluated the association between their expressions in nuclei, cytoplasms, or intraluminal secretions of benign/high-grade prostatic intraepithelial neoplasia/carcinoma cells and clinicopathologic profile available for our patient cohort. Stains were positive in 32%/77%/84% (nuclear semenogelin I), 87%/94%/84% (nuclear semenogelin II), 56%/64%/37% (nuclear eppin), 7%/15%/11% (cytoplasmic semenogelin I), 6%/11%/9% (cytoplasmic semenogelin II), 68%/74%/95% (cytoplasmic eppin), 97%/98%/13% (secreted semenogelin I), 98%/97%/11% (secreted semenogelin II), and 97%/98%/48% (secreted eppin) of benign/prostatic intraepithelial neoplasia/carcinoma, respectively. The levels of nuclear semenogelin I/cytoplasmic eppin were significantly higher in carcinoma than in benign ($P < .001/P < .001$) or prostatic intraepithelial neoplasia ($P < .001/P < .001$) and in prostatic intraepithelial neoplasia than in benign ($P < .001/P = .006$). Significantly higher nuclear semenogelin II expression was found in prostatic intraepithelial neoplasia than in benign ($P < .001$) or carcinoma ($P < .001$). Significantly lower nuclear eppin expression was seen in carcinoma than in benign ($P < .001$) or prostatic intraepithelial neoplasia ($P < .001$). Secreted semenogelin I, secreted semenogelin II, and secreted eppin were all significantly lower in carcinoma than in benign ($P < .001$) or prostatic intraepithelial neoplasia ($P < .001$). There were no statistically significant correlations between each stain and clinicopathologic features except significantly lower nuclear eppin expression in Gleason score 8 or higher tumors. Kaplan-Meier and log-rank tests further revealed that patients with nuclear semenogelin I–positive tumor had a significantly higher risk for biochemical recurrence ($P = .046$). Multivariate Cox model showed a trend toward significance ($P = .093$) in nuclear semenogelin I positivity as an independent predictor for recurrence. These results suggest that nuclear semenogelin I expression could be a reliable prognosticator in men who undergo radical prostatectomy.
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1. Introduction

Semenogelins I (SgI) (~50 kd consisting of 439 amino acids) and II (SgII) (~63 kd consisting of 559 amino acids with 78% homology to SgI) constitute the major structural

proteins in human semen and are well-known to contribute to sperm clotting [1]. After ejaculation, these proteins are degraded into smaller (5-20 kd) fragments by prostate-specific antigen (PSA), resulting in clotted gel liquefaction and release of the trapped spermatozoa [2]. SgI and SgII, upon binding to Zn^{2+} present in semen, have also been shown to inhibit the protease activity of PSA [3]. SgI and SgII are highly expressed in the seminal vesicles as well as in other male genital organs such as vas deferens, epididymis, and prostate [4]. In addition, previous studies showed semenogelin expression in nongenital organs such as the trachea, salivary gland, pancreas, kidney, and retina [4,5], suggesting a physiologic role of these proteins as modulators of zinc-dependent proteases throughout the body [1,2].

Eppin (epidermal protease inhibitor) is mainly expressed and secreted in the epididymis and testis [6]. It has been identified in a protein complex containing SgI in seminal plasma and on the surface of spermatozoa, resulting in inhibition of sperm motility [6,7]. Clinical use of antieppin antibodies has been evaluated for male contraception [6].

Thus, physiologic functions of semenogelins and eppin in male reproductive organs have been thoroughly studied. In contrast, the biologic role of these seminal plasma proteins in malignancies, including prostate cancer, is poorly understood. A study by Lundwall et al [4] demonstrated the expression of SgI and SgII in a prostate cancer cell line LNCaP as well as in a single case of human prostate cancer. We recently confirmed these findings and further showed increased expression of SgI and SgII in prostate cancer tissues compared with corresponding benign prostate [8]. However, perhaps because of small sample size (n = 70) with a relatively short follow-up duration (mean, 29.2 months) in the study [8], prognostic significance of semenogelin expression was unlikely conclusive. We separately showed that the levels of eppin expression were elevated in the 70 prostate cancers than in nonneoplastic prostate glands [9].

In the current study, we aim to validate our earlier results in a larger patient cohort with longer follow-up. This would be the first large study to simultaneously investigate the expression (nuclear, cytoplasmic, and intraluminal) of SgI, SgII, and eppin in benign prostate, prostatic intraepithelial neoplasia (PIN), and prostate cancer tissues.

2. Materials and methods

2.1. Tissue samples

We retrieved 291 prostate specimens obtained by radical prostatectomy performed at the University of Rochester Medical Center. Appropriate approval from the institutional review board at our institution was obtained before construction and use of the tissue microarray (TMA). Prostate TMAs were constructed from each representative lesion (benign, high-grade PIN, and carcinoma), as previously described [8]. These TMAs included PIN lesions only from 176 cases. The mean age of the patients at presentation was 60.4 years (range, 42-78 years), and the mean follow-up after the surgery was 47.5 months (range, 3-100 months). None of the patients had received therapy with hormonal reagents, radiation, or other anticancer drugs pre- or postoperatively before clinical or biochemical recurrence. *Biochemical recurrence* was defined as a single PSA level of 0.2 ng/mL or more.

2.2. Immunohistochemistry

Immunohistochemical staining was performed on the automated staining system, as described previously [8,9]. Briefly, the sections (4-μm thick) were immunohistochemically labeled using the primary antibodies to SgI (ab47142, dilution 1:1000; Abcam, Cambridge, MA), SgII (ab47141, dilution 1:1000; Abcam), and eppin (H-100, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate positive (human seminal vesicle or testis tissue) and negative (nonimmune rabbit immunoglobin G) controls were used concurrently. All these stains were manually scored by 1 pathologist (H. M.) blinded to patient identity. German Immunoreactive Score was calculated by multiplying the percentage of immunoreactive cells (0%, 0; 1%-10%, 1; 11%-50%, 2; 51%-80%, 3; and 81%-100%, 4) by staining intensity (negative, 0; weak, 1; moderate, 2; and strong, 3). Cores with the immunoreactive score of 0 or 1 were considered negative (0), and those with the immunoreactive scores of 2 to 4, 6 to 8, and 9 to 12 were considered weakly positive (1+), moderately positive (2+), and strongly positive (3+), respectively.

2.3. Statistical analyses

The Fisher exact and the χ^2 tests were used to evaluate the association between categorized variables. Nonparametric 2- and multigroup comparisons were carried out using Mann-Whitney U and Kruskal-Wallis tests, respectively, to assess differences in variables with ordered distribution across dichotomous categories. Survival rates in patients were calculated by the Kaplan-Meier method, and comparison was made by log-rank test. Correlation analyses were performed using the Spearman correlation test. The Cox proportional hazards model was used to determine statistical significance of predictors in a multivariate setting. $P < .05$ was considered to be statistically significant.

3. Results

3.1. Immunoreactivity in benign, PIN, and carcinoma cells

We immunohistochemically investigated nuclear (n-) and cytoplasmic (c-) expressions of SgI, SgII, and eppin in 291

cases of prostatic carcinoma as well as corresponding PINs (176 cases) and benign prostatic epithelia (291 cases) (Table 1). Positive signals for SgI and SgII were detected predominantly in nuclei of epithelial/carcinoma cells, whereas those for eppin were observed mainly in their cytoplasms (Fig. 1).

n-SgI was positive in 94 of 291 (32%; all weak; mean score, 1.41) benign, 136 of 176 (77%; 118 weak and 18 moderate; mean score, 2.50) PIN, and 245 of 291 (84%; 159 weak, 72 moderate, and 14 strong; mean score, 3.45) carcinoma tissues. Thus, n-SgI expression was significantly stronger in carcinoma than in benign ($P < .001$) or PIN ($P < .001$) and in PIN than in benign ($P < .001$). c-SgI was positive in 20 of 291 (7%; all weak; mean score, 0.91) benign, 26 of 176 (14%; 25 weak and 1 moderate; mean score, 1.05) PIN, and 31 of 291 (11%; all weak; mean score, 0.94) carcinoma tissues. There were no statistically significant differences in c-SgI expression among benign, PIN, and carcinoma.

n-SgII was positive in 253 of 291 (87%; 219 weak, 32 moderate, and 2 strong; mean score, 2.99) benign, 165 of 176 (94%; 78 weak, 77 moderate, and 10 strong; mean score, 4.35) PIN, and 244 of 291 (84%; 200 weak, 42 moderate, and 2 strong; mean score, 3.10) carcinoma tissues. Thus, n-SgII expression was significantly stronger in PIN than in benign ($P < .001$) or carcinoma ($P < .001$), whereas no significant difference in n-SgII was seen between benign and carcinoma ($P = .126$). c-SgII was positive in 18 of 291 (6%; all weak; mean score, 0.98) benign, 19 of 176 (11%; all weak; mean score, 0.98) PIN, and 25 of 291 (9%; all weak; mean score, 1.01) carcinoma tissues. There were no statistically significant differences in c-SgII expression among benign, PIN, and carcinoma.

n-Eppin was positive in 162 of 291 (56%; 147 weak and 15 moderate; mean score, 2.06) benign, 113 of 176 (64%; 112 weak and 1 moderate; mean score, 1.98) PIN, and 108 of 291 (37%; 106 weak and 2 moderate; mean score, 1.59) carcinoma tissues. Thus, n-eppin expression was significantly weaker in carcinoma than in benign ($P < .001$) or PIN ($P < .001$), whereas no significant difference in n-eppin was seen between benign and PIN ($P = .688$). c-Eppin was positive in 198 of 291 (68%; 143 weak, 41 moderate, and 14 strong; mean score, 2.84) benign, 131 of 176 (74%; 81 weak, 32 moderate, and 18 strong; mean score, 3.42) PIN, and 277 of 291 (95%; 120 weak, 107 moderate, and 50 strong; mean score, 4.64) carcinoma tissues. Thus, c-eppin expression was significantly stronger in carcinoma than in benign ($P < .001$) or PIN ($P < .001$) and in PIN than in benign ($P = .006$).

Next, we analyzed the correlations of eppin expression with the expression of semenogelins (Table 2). In benign tissues, there were weak positive correlations (correlation coefficient, 0.2-0.4) between n-eppin versus n-SgI or n-SgII and between c-eppin versus c-SgI or c-SgII. In PIN tissues, there were also weak positive correlations between n-eppin versus n-SgII as well as c-eppin versus c-SgI or c-SgII but not between n-eppin versus n-SgI. In carcinoma tissues,

Table 1 Expression of SgI, SgII, and eppin in prostate TMAs

|	n	SgI 0 (0-1) (%)	1+ (2-4) (%)	2+ (6-8) (%)	3+ (9-12) (%)	Score (mean ± SD)	P	SgII 0 (0-1) (%)	1+ (2-4) (%)	2+ (6-8) (%)	3+ (9-12) (%)	Score (mean ± SD)	P	Eppin 0 (0-1) (%)	1+ (2-4) (%)	2+ (6-8) (%)	3+ (9-12) (%)	Score (mean ± SD)	P
Nucleus							<.001						<.001						<.001
Benign	291	197 (68)	94 (32)	0 (0)	0 (0)	1.41 ± 0.73		38 (13)	219 (75)	32 (11)	2 (1)	2.99 ± 1.23		129 (44)	147 (51)	15 (5)	0 (0)	2.06 ± 1.24	
PIN	176	40 (23)	118 (67)	18 (10)	0 (0)	2.50 ± 1.20		11 (6)	78 (44)	77 (44)	10 (6)	4.35 ± 1.55		63 (36)	112 (64)	1 (1)	0 (0)	1.98 ± 0.85	
Carcinoma	291	46 (16)	159 (55)	72 (25)	14 (5)	3.45 ± 1.75		47 (16)	200 (69)	42 (14)	2 (1)	3.10 ± 1.35		183 (63)	106 (36)	2 (1)	0 (0)	1.59 ± 0.97	
Cytoplasm							.057						.658						<.001
Benign	291	271 (93)	20 (7)	0 (0)	0 (0)	0.91 ± 0.65		273 (94)	18 (6)	0 (0)	0 (0)	0.98 ± 0.58		93 (32)	143 (49)	41 (14)	14 (5)	2.84 ± 1.98	
PIN	176	150 (85)	25 (14)	1 (1)	0 (0)	1.05 ± 0.74		157 (89)	19 (11)	0 (0)	0 (0)	0.98 ± 0.57		45 (26)	81 (46)	32 (18)	18 (10)	3.42 ± 2.20	
Carcinoma	291	260 (89)	31 (11)	0 (0)	0 (0)	0.94 ± 0.66		266 (91)	25 (9)	0 (0)	0 (0)	1.01 ± 0.55		14 (5)	120 (41)	107 (37)	50 (17)	4.64 ± 2.08	
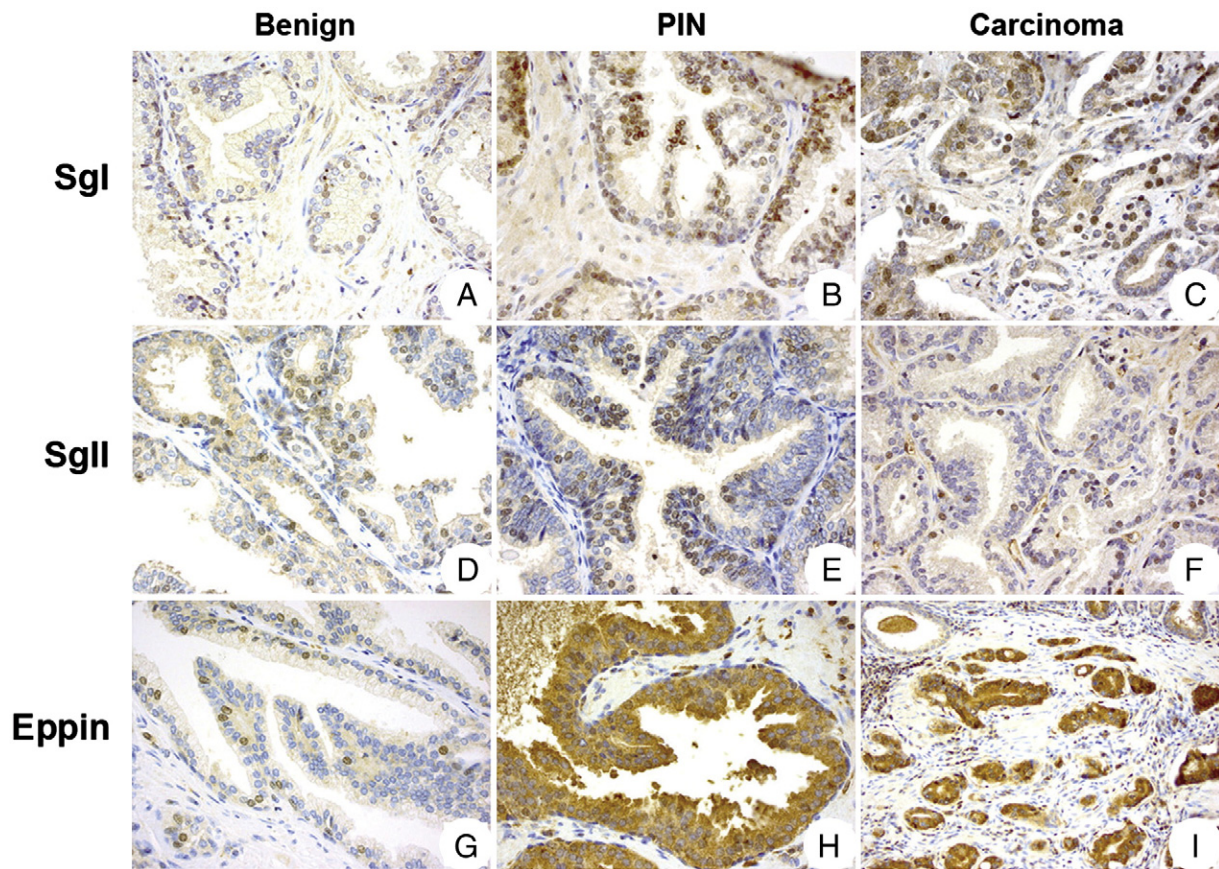
Fig. 1 Immunohistochemistry of SgI (A-C), SgII (D-F), and eppin (G-I) in benign (A, D, and G), PIN (B, E, and H), and cancerous (C, F, and I) prostate tissue samples. Original magnification ×400 (A-H) or ×200 (I).

weak positive correlation was only seen between n-eppin versus n-SgII.

3.2. Cellular immunoreactivity and clinicopathologic features

We further evaluated the correlation of expression levels of respective stains with clinicopathologic profile available for our patient cohort. Table 3 summarizes the correlations between the expression of n-SgI, n-SgII, n-eppin, or c-eppin

in carcinoma and Gleason score (GS), pathologic stage (pT), lymph node metastasis, or preoperative PSA. Significantly lower n-eppin expression was observed in GS 8 or higher tumors (versus GS ≤7, P = .044), but there were no statistically significant correlations between each stain and other clinicopathologic features. It was noted that preoperative PSA levels were significantly higher (P = .017) when compared between n-SgI–positive (mean ± SD, 6.66 ± 4.44 ng/mL) versus n-SgI–negative (mean ± SD, 4.78 ± 1.90 ng/mL) tumors, but they were only slightly higher in patients with n-SgII–positive/n-eppin–positive/c-eppin–positive tumors (P = .752/P = .688/P = .717, respectively).

We then performed Kaplan-Meier analysis coupled with log-rank test to assess possible associations between each staining and disease progression. Of the 291 patients with a mean follow-up of 47.5 months, 33 (11%) had a clinical or biochemical recurrence after radical prostatectomy. Of these, 31 cases (94%) exhibited GS 7 or higher, extraprostatic extension (pT3a), seminal vesicle involvement (pT3b), and/or lymph node metastasis (pN1). Higher expression levels of n-SgI significantly correlated (0 versus 3+, P < .001; 1+ versus 3+, P = .003) or tended to correlate (0 versus 1+, P = .079; 0 versus 2+, P = .064; 2+ versus 3+, P = .078) with recurrence (Fig. 2A). Thus, patients with n-SgI–positive tumor had a significantly higher risk of recurrence compared

Table 2 Correlation between eppin and semenogelin expression

Nucleus		Eppin		Cytoplasm		Eppin	
		CC	P			CC	P
Benign	SgI	0.386	<.001	Benign	SgI	0.220	<.001
	SgII	0.308	<.001		SgII	0.215	<.001
PIN	SgI	0.198	.009	PIN	SgI	0.296	<.001
	SgII	0.250	<.001		SgII	0.231	.002
Carcinoma	SgI	0.192	.001	Carcinoma	SgI	0.063	.283
	SgII	0.290	<.001		SgII	-0.009	.878
Abbreviation: CC, correlation coefficient.							

Table 3 Correlation between the expression of nuclear SgI, nuclear SgII, nuclear eppin, or cytoplasmic eppin and clinicopathologic profile of the patients

	n	SgI (nuclear staining)						SgII (nuclear staining)					
		0 (0-1) (%)	1+ (2-4) (%)	2+ (6-8) (%)	3+ (9-12) (%)	Score (mean ± SD)	P	0 (0-1) (%)	1+ (2-4) (%)	2+ (6-8) (%)	3+ (9-12) (%)	Score (mean ± SD)	P
GS							.370						.080
≤6	104	20 (19)	59 (57)	21 (20)	4 (4)	3.24 ± 1.65		21 (20)	67 (64)	15 (14)	1 (1)	2.94 ± 1.43	
7	170	25 (15)	88 (52)	48 (28)	9 (5)	3.56 ± 1.79		24 (14)	118 (69)	27 (16)	1 (1)	3.24 ± 1.33	
≥8	17	1 (6)	12 (71)	3 (18)	1 (6)	3.59 ± 1.88		2 (12)	15 (88)	0 (0)	0 (0)	2.68 ± 0.76	
pT							.224						.898
2	229	35 (15)	130 (57)	55 (24)	9 (4)	3.39 ± 1.69		40 (17)	154 (67)	34 (15)	1 (0)	3.07 ± 1.37	
3a	48	10 (21)	23 (48)	11 (23)	4 (8)	3.45 ± 1.92		6 (13)	34 (71)	7 (15)	1 (2)	3.21 ± 1.34	
3b	14	1 (7)	6 (43)	6 (43)	1 (7)	4.29 ± 1.97		1 (7)	12 (86)	1 (7)	0 (0)	3.24 ± 1.29	
Lymph node metastasis (pN)							.118						.668
0	137	16 (12)	78 (57)	38 (28)	5 (4)	3.52 ± 1.62		17 (12)	102 (74)	17 (12)	1 (1)	3.16 ± 1.27	
1	7	0 (0)	3 (43)	3 (43)	1 (14)	4.79 ± 2.27		0 (0)	6 (86)	1 (14)	0 (0)	3.19 ± 1.42	
Preoperative PSA (ng/mL)							.429						.429
<4.0	49	8 (16)	27 (55)	10 (20)	4 (8)	3.41 ± 2.08		10 (20)	33 (67)	6 (12)	0 (0)	2.95 ± 1.50	
4.0-10.0	178	25 (14)	96 (54)	47 (26)	10 (6)	3.57 ± 1.70		25 (14)	121 (68)	30 (17)	2 (1)	3.19 ± 1.35	
≥10.0	25	0 (0)	16 (64)	9 (36)	0 (0)	3.69 ± 1.22		6 (24)	15 (60)	4 (16)	0 (0)	3.04 ± 1.50	

|		Eppin (nuclear staining)						Eppin (cytoplasmic staining)					
		0 (0-1) (%)	1+ (2-4) (%)	2+ (6-8) (%)	3+ (9-12) (%)	Score (mean ± SD)	P	0 (0-1) (%)	1+ (2-4) (%)	2+ (6-8) (%)	3+ (9-12) (%)	Score (mean ± SD)	P
GS							.021						.522
≤6	104	71 (68)	32 (31)	1 (1)	0 (0)	1.52 ± 0.98		5 (5)	41 (39)	34 (33)	24 (23)	4.79 ± 2.15	
7	170	99 (58)	70 (41)	1 (1)	0 (0)	1.69 ± 0.96		9 (5)	72 (42)	67 (39)	22 (13)	4.53 ± 1.97	
≥8	17	13 (76)	4 (24)	0 (0)	0 (0)	1.08 ± 0.79		0 (0)	7 (41)	6 (35)	4 (24)	4.88 ± 2.26	
pT							.193						.692
2	229	139 (61)	88 (38)	2 (1)	0 (0)	1.64 ± 0.98		9 (4)	94 (41)	86 (38)	40 (17)	4.65 ± 2.03	
3a	48	36 (75)	12 (25)	0 (0)	0 (0)	1.33 ± 0.84		4 (8)	22 (46)	14 (29)	8 (17)	4.55 ± 2.26	
3b	14	8 (57)	6 (43)	0 (0)	0 (0)	1.70 ± 1.12		1 (7)	4 (29)	7 (50)	2 (14)	4.89 ± 1.75	
Lymph node metastasis (pN)							.806						.797
0	137	93 (68)	43 (31)	1 (1)	0 (0)	1.52 ± 0.99		6 (4)	59 (43)	52 (38)	20 (15)	4.45 ± 1.94	
1	7	4 (57)	3 (43)	0 (0)	0 (0)	1.44 ± 1.14		0 (0)	3 (43)	3 (43)	1 (14)	4.50 ± 1.58	
Preoperative PSA (ng/mL)							.680						.323
<4.0	49	31 (63)	18 (37)	0 (0)	0 (0)	1.52 ± 1.02		3 (6)	24 (49)	14 (29)	8 (16)	4.45 ± 2.17	
4.0-10.0	178	114 (64)	62 (35)	2 (1)	0 (0)	1.62 ± 0.98		5 (3)	70 (39)	72 (40)	31 (17)	4.75 ± 1.93	
≥10.0	25	16 (64)	9 (36)	0 (0)	0 (0)	1.42 ± 0.85		2 (8)	11 (44)	8 (32)	4 (16)	4.20 ± 2.06	
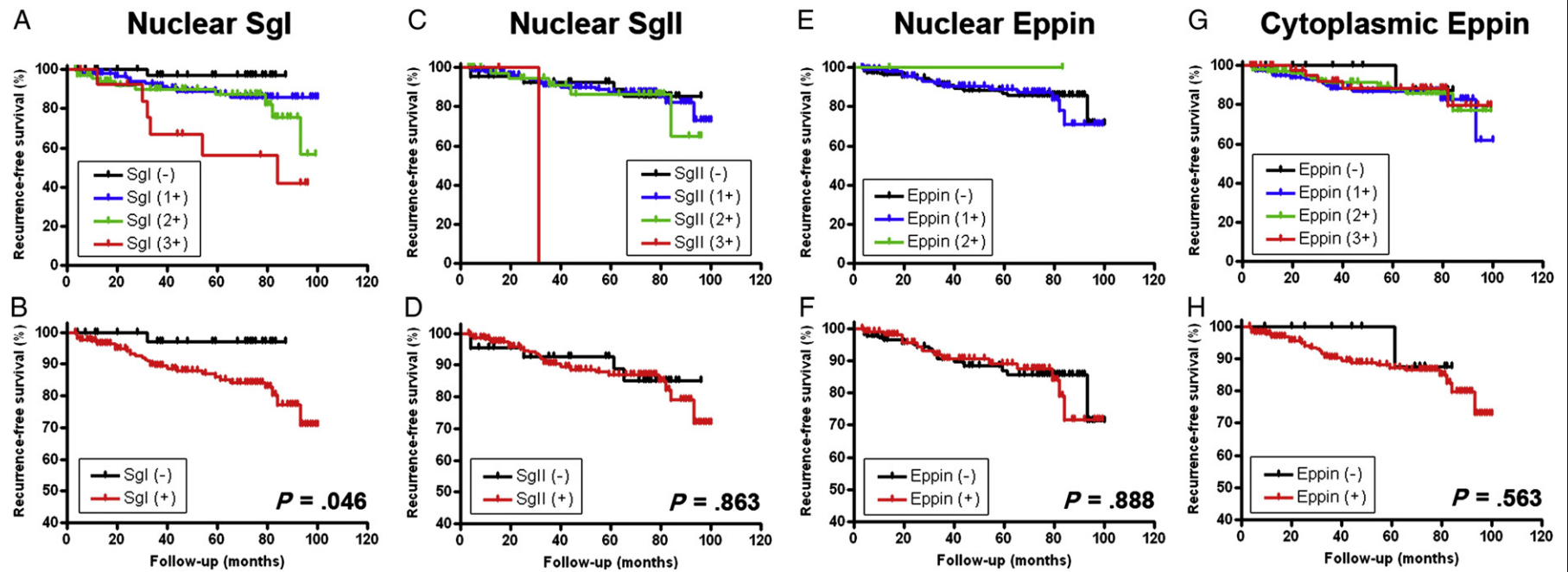
Fig. 2 Kaplan-Meier analysis of recurrence-free survival according to the expression levels of nuclear SgI (A and B), nuclear SgII (C and D), nuclear eppin (E and F), and cytoplasmic eppin (G and H).

Table 4 Multivariate Cox model for biochemical recurrence

	HR	95% CI	P
GS [a]	4.52	1.36-15.02	.014
Pathologic stage [b]	2.50	1.24-5.01	.010
SgI [c]	5.53	0.75-40.56	.093

Abbreviations: HR, hazards ratio; CI, confidence interval.

[a] GS 6 or lower versus 7 or higher.

[b] pT2N0 versus pT3 and/or pN1.

[c] Nuclear SgI negative versus positive.

with those with n-SgI–negative tumor (P = .046) (Fig. 2B). However, the expression status of n-SgII (Fig. 2C-D), n-eppin (Fig. 2E-F), or c-eppin (Fig. 2G-H) showed no strong correlation with recurrence.

To see whether n-SgI expression was an independent predictor of recurrence, multivariate analysis was performed with Cox model, including dichotomized n-SgI expression (positive versus negative), GS ($\leq$6 versus $\geq$7), and pT (pT2N0 versus pT3 and/or pN1) (Table 4). Positivity of n-SgI was found to show a trend toward significance (P = .093).

3.3. Immunoreactivity in intraluminal secretions

In addition to cellular expression, secreted materials (s-) were also assessed (Table 5) because semenogelins and eppin are essentially secreted proteins. Moderate to strong immunoreactivity to SgI, SgII, and eppin was seen in most (97%-98%) benign or PIN glands where the secretions were present (Fig. 3). In contrast, intraluminal dense amorphous acellular secretions often identified in carcinomas but only

occasionally seen in benign glands [10] were uncommonly immunoreactive to SgI (13%), SgII (11%), and eppin (48%), and their signals, if present, were mostly weak. Thus, s-SgI, s-SgII, and s-eppin were significantly lower in carcinoma than in benign (all P < .001) and PIN (all P < .001). In addition, the status of s-SgI, s-SgII, or s-eppin was not associated with each clinicopathologic feature (Table 5). Interestingly, none of the patients with s-SgI–positive or s-SgII–positive tumor developed recurrence. However, positivity of s-SgI (Fig. 4A, P = .141), s-SgII (Fig. 4B, P = .177), or s-eppin (Fig. 4C, P = .481) did not significantly correlate with recurrence-free survival.

We also assessed the correlation of cellular (nucleus and cytoplasm) and intraluminal expression (data not shown). There were no significant associations of the expression levels among the 3 locations.

4. Discussion

In contrast to well-recognized physiologic functions of semenogelins and eppin in the male reproductive system [1-7], the role of these seminal plasma proteins in human malignancies remains unclear. Only a few studies have shown the expression of semenogelins in malignancies, including lung carcinomas, melanoma, and leukemias [11,12]. Recently, in 70 cases of radical prostatectomy, we showed that expression levels of n-SgI and n-SgII were elevated in carcinoma than in corresponding benign tissues and that men with SgI-positive/SgII-negative tumor had a significantly higher risk of recurrence [8]. Furthermore, in

Table 5 Intraluminal expression of SgI, SgII, and eppin in prostate TMAs

|	SgI		SgII		Eppin	
	Positive/total (%)	P	Positive/total (%)	P	Positive/total (%)	P
		<.001		<.001		<.001
Benign	202/209 (97)		211/215 (98)		207/214 (97)	
PIN	86/88 (98)		85/88 (97)		88/90 (98)	
Carcinoma	13/101 (13)		12/105 (11)		49/102 (48)	
GS		.548		.211		.661
$\leq$6	8/47 (17)		9/51 (18)		23/44 (52)	
7	5/48 (10)		3/48 (6)		24/52 (46)	
$\geq$8	0/6 (0)		0/6 (0)		2/6 (33)	
pT		.820		.701		.142
2	12/82 (15)		10/85 (12)		42/90 (47)	
3a	1/15 (7)		1/14 (7)		7/10 (70)	
3b	0/4 (0)		1/6 (17)		0/2 (0)	
Lymph node metastasis (pN)		1.000		1.000		.489
0	4/43 (9)		5/43 (12)		23/45 (51)	
1	0/3 (0)		0/3 (0)		0/2 (0)	
Preoperative PSA (ng/mL)		.850		.734		.545
<4.0	2/18 (11)		1/20 (5)		11/19 (58)	
4.0-10.0	7/64 (11)		7/63 (11)		29/61 (48)	
$\geq$10.0	1/6 (17)		1/8 (13)		2/6 (33)	
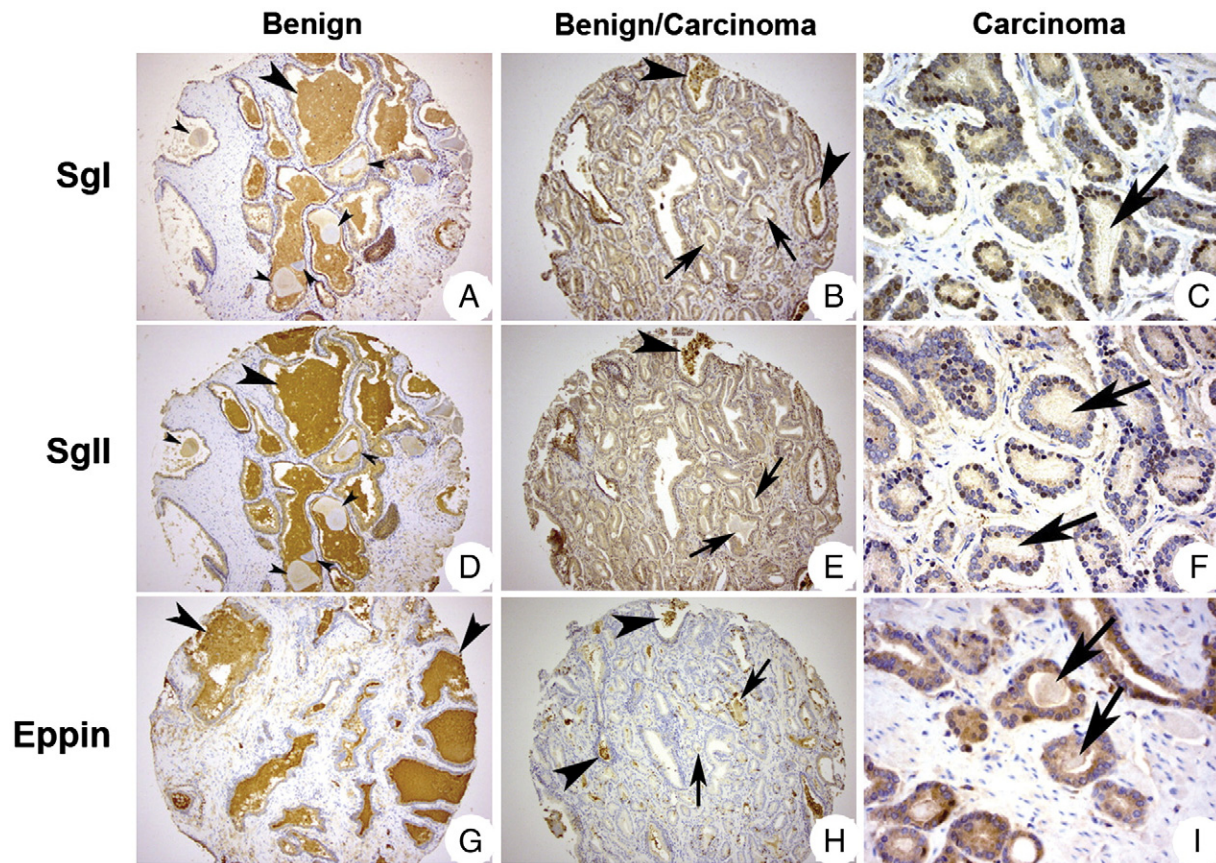
Fig. 3 Immunoreactivity to SgI (A-C), SgII (D-F), and eppin (G-I) in secreted materials. Large arrowheads indicate secretions in nonneoplastic glands; small arrowheads, corpora amylacea; arrows, dense amorphous secretions within cancer glands. Original magnification ×100 (A, B, D, E, G, and H) or ×400 (C, F, and I).

our previous study [8], SgI was found to promote the growth of androgen receptor–positive prostate cancer cells, whereas SgII marginally inhibited it. We also reported overexpression of c-eppin in the 70 cases of prostate cancer and its inverse correlation with the risk of biochemical recurrence [9]. The current study analyzed separately nuclear, cytoplasmic, and intraluminal expressions of SgI, SgII, and eppin in a larger number of samples, including nonneoplastic prostate, PIN, and prostatic carcinoma.

In accordance with our earlier report [8], n-SgI was significantly higher in carcinoma than in benign tissues. We, in addition, revealed that n-SgI was significantly higher in PIN than in benign and in carcinoma than in PIN, suggesting its role as a promoter in prostate carcinogenesis. In contrast, inconsistent with our previous findings [8], expression levels of n-SgII were similar between benign and carcinoma tissues. Accordingly, SgII may play a minor role in prostate cancer. However, because significantly higher expression of n-SgII was seen in PIN than in benign and carcinoma, SgII
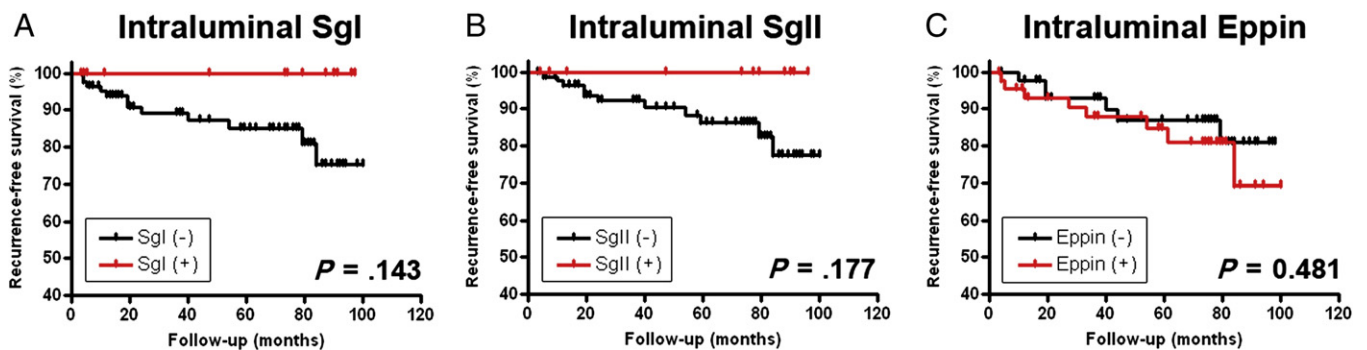
Fig. 4 Kaplan-Meier analysis of recurrence-free survival according to the expression levels of intraluminal SgI (A), SgII (B), and eppin (C).

might still involve an early event of prostate tumorigenesis. Although we had failed to find statistically significant associations between the status of SgI or SgII expression alone and recurrence [8], n-SgI positivity alone, but not combinations of n-SgI and n-SgII positivity (data not shown) or n-SgII status alone, precisely predicted recurrence in the current study. Nonetheless, n-SgI did not significantly correlate with tumor grades and stages, implying that SgI could be an independent predictor of tumor progression. Indeed, multivariate analysis revealed a tendency toward significance ($P = .093$) in n-SgI positivity as a predictor for recurrence. Furthermore, preoperative serum levels of PSA were significantly higher in patients with n-SgI–positive tumor than in those with n-SgI–negative tumor, suggesting SgI induces PSA secretion from prostate cancer. Because PSA physiologically degrades semenogelins in semen [1,2], it is possible that the increase in PSA levels may, at least partially, represent an attempt to target semenogelins rather than tumor progression.

Consistent with our earlier observation [9], c-eppin was higher in carcinoma than in benign or PIN and in PIN than in benign. Oppositely to c-eppin results, n-eppin was lower in carcinoma than in benign and PIN. These results may suggest that subcellular localization of eppin affects prostate cancer development. Although we previously demonstrated an inverse correlation of c-eppin expression with biochemical recurrence [9], neither n-eppin nor c-eppin showed such correlations in this larger study.

Because eppin is shown to bind to semenogelin in seminal plasma to inhibit sperm motility [6,7], we assessed if eppin coexpressed with semenogelins in prostate tissues. Eppin-SgI and eppin-SgII showed weak positive correlations in both nucleus and cytoplasm of nonneoplastic prostate and PIN (except n-eppin and n-SgI) glands. Interestingly, their correlations were lost in carcinoma cells except a weak positive correlation of n-eppin and n-SgII. These results suggest that eppin and semenogelins (especially SgI) dissociate during cancer development.

Intraluminal expressions of SgI, SgII, and eppin were all dramatically reduced in carcinoma compared with benign and PIN glands. Indeed, we failed to detect semenogelin signals in the conditioned medium after culturing semenogelin-positive LNCaP cells as well as 3 other semenogelin-negative prostate cancer cell lines by Western blotting (unpublished data). Immunoreactivity to SgI, SgII, or eppin in dense secretions within cancer glands in the current study was not associated with PSA levels, tumor grade/stage, and recurrence. These results suggest that, in contrast to benign or PIN cells, carcinoma cells do not generally secrete a large amount of these proteins. It has been known that the secretions similar to those seen in benign glands, which may contain spermatozoa, are rarely found in cancer glands [13]. Thus, although semenogelins and eppin, as secreted proteins, can be found in seminal plasma, their expression pattern in carcinoma becomes more localized within the cells. It was noted that none of the patients with intraluminal SgI- or SgII-

positive tumor had recurrence. Therefore, not only n-SgI positivity but also SgI localization can be a potential predictor of recurrence after radical prostatectomy. As noted, secreted forms of these proteins should be able to be detected in semen as well as in blood or urine, and increased serum semenogelins were detected in patients with lung cancer [14]. Additional studies, including assessment of seminal plasma proteins in body fluids from patients with prostate cancer, might validate these as biomarkers of prostate cancer detection and tumor progression.

In conclusion, significant increases in the expression of n-SgI and c-eppin as well as a significant decrease in n-eppin expression were observed in prostate cancer compared with benign or PIN glands. Higher expression of n-SgII in PIN than in benign or cancerous prostate was also noted. Moreover, n-SgI expression was suggested to be a reliable biomarker for prostate cancer recurrence after radical prostatectomy. In addition, we previously showed that semenogelins were able to modulate prostate cancer cell proliferation [8]. These results might indicate the involvement of semenogelins and eppin in the development and progression of prostate cancer. Further functional analyses of these seminal plasma proteins are necessary to determine their biologic significance in prostate cancer.

Acknowledgment

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Epidermal growth factor induces bladder cancer cell proliferation through activation of the androgen receptor

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Abstract. Androgen receptor (AR) signals have been suggested to contribute to bladder tumorigenesis and cancer progression. Activation of epidermal growth factor receptor (EGFR) also leads to stimulation of bladder tumor growth. However, crosstalk between AR and EGFR pathways in bladder cancer remains uncharacterized. We have recently shown that androgens activate the EGFR pathway in bladder cancer cells. The purpose of this study was to investigate the effects of EGF on AR activity in bladder cancer. EGF increased AR transcriptional activity by 1.2-, 1.9- and 2.0-fold in UMUC3, 5637-AR and J82-AR cell lines, respectively, over mock treatment and a specific EGFR inhibitor, PD168393, antagonized the EGF effect. Combined treatment of EGF and dihydrotestosterone (DHT) further induced AR transactivation while an AR antagonist, hydroxyflutamide (HF), abolished the effect of not only DHT but also EGF. In growth assays, EGF alone/DHT alone/EGF+DHT increased cell numbers by 16/12/19%, 6/14/18% and 30/12/38% in UMUC3-control-shRNA, 5637-AR and J82-AR, respectively, whereas the effects of EGF were marginal or less significant in UMUC3-AR-shRNA (8%) or AR-negative 5637-V (<1%) and J82-V (17%) cells. HF treatment at least partially counteracted the EGF effect on the growth of AR-positive cells. Western blotting demonstrated that EGF, especially in the presence of DHT, upregulated the expression of the p160 coactivator TIF2

and HF again blocked this stimulation. Co-immunoprecipitation revealed the association between AR and estrogen receptor (ER)-β or Src in UMUC3 cells and stronger associations with EGF treatment, implying the involvement of the AR/ER/Src complex in EGF-increased AR transactivation and cell growth. Current results, thus, suggest that EGF promotes bladder cancer cell proliferation via modulation of AR signals. Taken together with our previous findings, crosstalk between EGFR and AR pathways can play an important role in the progression of bladder cancer.

Introduction

Epidemiological and clinical evidence has indicated a substantially higher risk of urinary bladder cancer in males yet there is a tendency showing more aggressive behavior in tumors from female patients (1,2). Recent experimental data suggest that urothelial carcinoma, like prostate and breast cancers, is an endocrine-related neoplasm (reviewed in ref. 3). In particular, the androgen receptor (AR) and estrogen receptor (ER) signaling pathways have been shown to contribute to bladder tumorigenesis and cancer progression (3-13), which may explain some of the differences in male versus female bladder cancer.

Activation of the epidermal growth factor (EGF) receptor (EGFR) family is known to involve the growth and progression of a variety of malignancies. In bladder cancer, EGFR/ERBB2 is frequently overexpressed, which correlates with higher tumor grade/stage and poorer prognosis (14-16). Experimental evidence in bladder cancer has also suggested that the EGFR pathway plays a critical role in cell proliferation, apoptosis, differentiation, migration and angiogenesis (17-19). Consequently, the efficacy of targeted therapy directed at EGFR signals has been assessed in bladder cancer.

The crosstalk between nuclear hormone receptors and growth factors leads to activation of nuclear receptor-mediated transcription. Specifically, in prostate cancer cells, AR signals upregulate *EGFR* and *ERBB2* gene expression, whereas activation of EGFR and ERBB2 modulates AR functions (20-24). It has also been shown that the assembly of the EGFR/AR/ER/Src signaling complex is crucial for proliferation of prostate and breast cancer cells triggered by androgens, estrogens and/or EGF (25). In contrast, the relationship between the AR and EGFR pathways in bladder cancer remains poorly understood. We have recently shown that AR activation results in upregula-

tion of EGFR and ERBB2 expression in bladder cancer cells, which may play an important role in androgen-mediated tumor progression (26). In the present study, we investigated whether EGF could alter AR activity in bladder cancer cells.

Materials and methods

Cell culture and chemicals. Human bladder cancer cell lines, UMUC3, 5637 and J82, obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. Cells were cultured in phenol-red free medium supplemented with 5% charcoal-stripped FBS at least 18 h before experimental treatment. We obtained dihydrotestosterone (DHT) and EGF from Sigma (St. Louis, MO, USA); hydroxyflutamide (HF) from Schering (Kenilworth, NJ, USA); and PD168393 from Calbiochem (San Diego, CA, USA).

Stable cell lines with AR and AR-short hairpin RNA (shRNA). Cell lines stably expressing a full-length wild-type human AR (5637-AR and J82-AR) or vector only (5637-V and J82-V) were established, using a lentivirus vector (pWPI-AR or pWPI-control) with psPAX2 envelope and pMD2.G packaging plasmids, as we described previously (11,26). Similarly, stable AR knockdown/control cell lines (UMUC3-AR-shRNA/UMUC3-control-shRNA) were established with a retrovirus vector pMSCV/U6-AR-shRNA or pMSCV/U6-control-shRNA (5,26).

Reporter gene assay. Bladder cancer cells at a density of 50-60% confluence in 24-well plates were co-transfected with 250 ng of MMTV-luc reporter plasmid DNA and 2.5 ng of pRL-TK-luc plasmid DNA, using GeneJuice transfection reagent (Novagen, Gibbstown, NJ, USA). Six hours after transfection, the medium was replaced with one supplemented with 5% charcoal-stripped FBS containing ethanol or ligands (DHT, HF, EGF and/or PD168393) for 24 h. Cells were harvested, lysed and assayed for luciferase activity determined using a dual-luciferase reporter assay kit (Promega, Madison, WI, USA) and luminometer (TD-20/20; Turner BioSystems, Sunnyvale, CA, USA).

Cell proliferation assay. We used the MTT (methyl thiazolyl diphenyl tetrazolium bromide) assay to assess cell viability, as described previously (26,27). Briefly, cells ($3x10^3$) seeded in 96-well tissue culture plates were incubated with medium supplemented with charcoal-stripped FBS in the presence or absence of ligands (DHT, HF and EGF). The media were refreshed every 24 h. After 96 h of treatment, 10 μl MTT (Sigma) stock solution (5 mg/ml) was added to each well with 0.1 ml of medium for 4 h at 37°C. The medium was replaced with 100 μl DMSO followed by incubation for 5 min at room temperature. The absorbance was then measured at a wavelength of 570 nm with background subtraction at 655 nm.

Western blotting. Protein extraction and western blotting were performed, as described previously (27) with minor modifications. Briefly, equal amounts of protein (20 μg) obtained from cell extracts were separated in a 10% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to

polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) by electroblotting using a standard protocol. Specific antibody binding was detected, using an anti-AR antibody (clone N20; diluted 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-transcriptional intermediary factor 2 (TIF2) antibody (clone 29/TIF2; diluted 1:1,000; BD Bioscience, Franklin Lakes, NJ, USA), or an anti-GAPDH antibody (clone 6C5; diluted 1:1,000; Santa Cruz Biotechnology), with horseradish peroxidase detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, IL, USA).

Co-immunoprecipitation. UMUC3 cells were treated with mock (ethanol) or EGF for 24 h and protein (500 μg) from the cell lysates was incubated with 2 μg of anti-AR antibody (N20) or normal rabbit IgG (Santa Cruz Biotechnology) for 16 h at 4°C with agitation. To each sample we added 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology), incubated for 1 h and washed thrice with radio-immunoprecipitation assay buffer. Then, the complex was resolved on a 10% SDS-PAGE, transferred to the membrane and blotted with an anti-ERβ antibody (clone 14C8; diluted 1:500; Abcam, Cambridge, MA, USA) or an anti-v-Src antibody (clone 327; diluted 1:1,000; Calbiochem).

Statistical analysis. Student's t-test was used to analyze differences in relative luciferase activity and relative cell number between the two groups. P<0.05 was considered statistically significant.

Results

EGF mediates AR transactivation via EGFR. Because previous studies showed ligand-independent activation of AR transcription by EGF in prostate cancer cells (20-22), we first assessed the effects of EGF and a specific EGFR inhibitor PD168393 on AR transactivation in bladder cancer lines. In AR-positive UMUC3 and AR-negative 5637 and J82 with a full-length AR stably expressed by lentivirus, luciferase activity was determined in the cell extracts with transfection of a plasmid (MMTV-luc) containing an androgen response element (ARE) as a reporter of AR-mediated transcriptional activity. As shown in Fig. 1, EGF treatment increased luciferase activity by 1.2-, 1.9- and 2.0-fold in UMUC3 (p=0.013), 5637-AR (p=0.036) and J82-AR (p=0.050), respectively, over mock treatment. PD168393 showing only marginal activity (in UMUC3 and 5637-AR) or some agonist effect (1.5-fold in J82-AR) could antagonize the EGF effect on AR transcription. In AR-knockdown UMUC3-AR-shRNA and AR-negative lines (5637, 5637-V, J82 and J82-V), EGF and/or PD168393 showed marginal effects on AR transcriptional activity (data not shown). These results suggest that EGF induces AR transactivation via EGFR in an androgen-independent manner.

Antiandrogen blocks EGF-induced AR transactivation. We next assessed the effect of EGF, in conjunction with androgen and/or antiandrogen, on AR transcriptional activity in bladder cancer cells. As shown in Fig. 2A, DHT treatment increased AR transcription by 25% (lanes 1 vs. 3, p=0.032) and addition of EGF further induced it by 35% (lanes 1 vs. 4, p=0.001; lanes 3 vs. 4, p=0.103) in UMUC3. Interestingly, HF showing
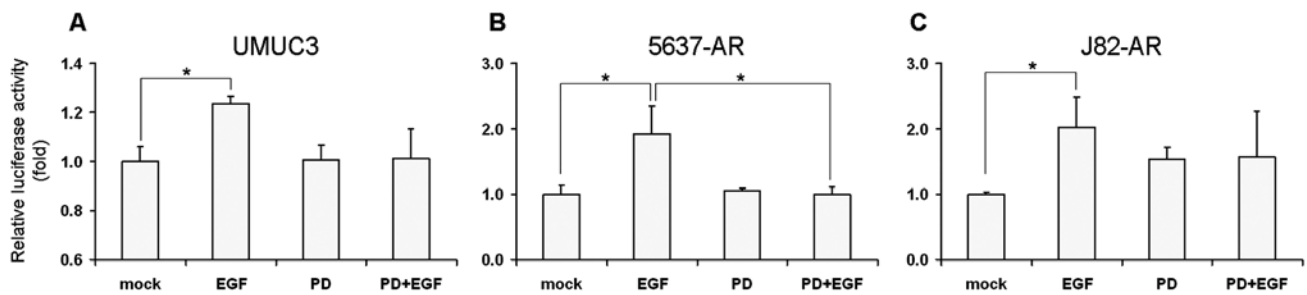
Figure 1. Effects of EGF on AR transactivation. Bladder cancer cells (A, UMUC3; B, 5637-AR; C, J82-AR) were transfected with MMTV-Luc and were then cultured for 24 h in the presence of ethanol (mock), 100 ng/ml EGF and/or 1 μM PD168393, as indicated. Luciferase activity analyzed in a luminometer is presented relative to that of mock treatment in each cell line (first lanes; set as 1-fold). Each value represents the mean + SD from at least three independent experiments. *p<0.05.
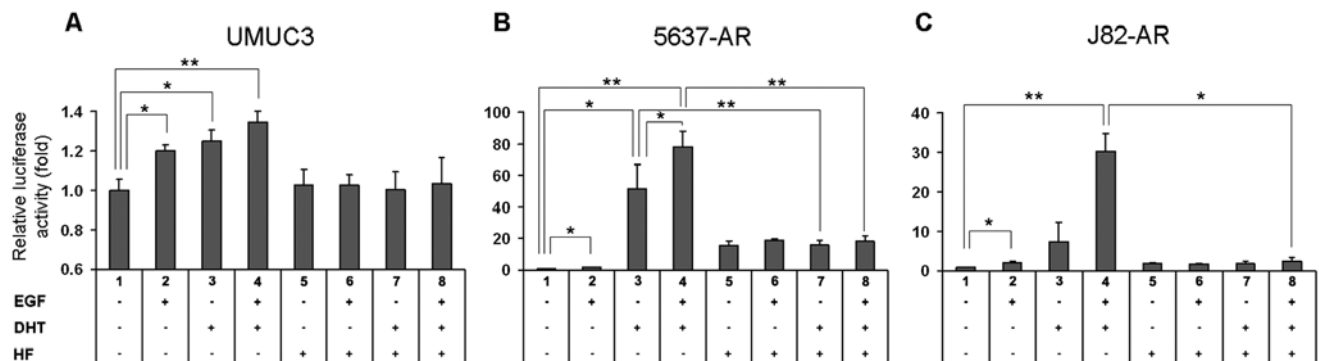

Figure 2. Effects of androgen and antiandrogen on EGF-mediated AR transactivation. Bladder cancer cells (A, UMUC3; B, 5637-AR; C, J82-AR) were transfected with MMTV-Luc and were then cultured for 24 h in the presence of ethanol (mock), 100 ng/ml EGF, 10 nM DHT and/or 10 μM HF, as indicated. Luciferase activity analyzed in a luminometer is presented relative to that of mock treatment in each cell line (first lanes; set as 1-fold). Each value represents the mean + SD from at least three independent experiments. *p<0.05; **p<0.01.

only marginal activity (lanes 1 vs. 5) abolished the effects of not only DHT (lanes 3 vs. 7, p=0.077) but also EGF (lanes 2 vs. 6, p=0.061) and EGF+DHT (lanes 4 vs. 8, p=0.082). Similarly, in 5637-AR (Fig. 2B) and J82-AR (Fig. 2C), DHT (lane 3) induced AR transcription to 52- and 7.4-fold, respectively and EGF in the presence of DHT (lanes 4 vs. 3) enhanced it to 78- (p=0.035) and 30-fold (p=0.054), respectively. HF showing some agonist activities (lanes 1 vs. 5) in 5637-AR (15-fold)/J82-AR (1.8-fold), which were much higher (vs. 1.7-fold)/similar (vs. 2.1-fold) compared to EGF stimulations (lane 2), could block the effects of DHT (lanes 3 vs. 7, p=0.005/p=0.164) and EGF+DHT (lanes 4 vs. 8, p=0.009/p=0.013). Again, in UMUC3-AR-shRNA, 5637(-V) and J82(-V) cells, EGF, DHT and/or HF showed marginal effects on AR transcription (data not shown). These findings suggest that EGF and androgen cooperatively induce AR transactivation that is sufficiently inhibited by an anti-androgen.

EGF stimulates cell growth via AR signaling. We then performed the MTT assay to investigate the effects of EGF androgen and antiandrogen on cell proliferation of bladder cancer lines with vs. without AR (i.e., UMUC3-control-shRNA vs. UMUC3-AR-shRNA, 5637-AR vs. 5637-V and J82-AR vs. J82-V). As shown in Fig. 3A, in UMUC3-control-shRNA, treatment of EGF, DHT and EGF+DHT increased cell growth by 16% (p=0.020), 12% (p=0.195) and 19% (p=0.009), respectively, over mock treatment and HF treatment appeared to restore the

growth to the basal levels. In UMUC3-AR-shRNA, DHT effect was marginal (2%) and the effects of EGF (8%, p=0.039) and EGF+DHT (11%, p=0.040) were less significant compared to those in UMUC3-control-shRNA. In 5637-AR, treatment of EGF, DHT and EGF+DHT induced cell growth by 6% (p=0.558), 14% (p=0.016) and 19% (p=0.050), respectively and HF almost completely abolished the stimulation (Fig. 3B). In 5637-V, only marginal effects of EGF, DHT and/or HF on cell numbers were seen. In J82-AR, treatment of EGF, DHT and EGF+DHT induced cell growth by 30% (p=0.001), 12% (p=0.179) and 38% (p<0.001), respectively (Fig. 3C). Interestingly, HF was able to antagonize the DHT effect but only partially blocked the EGF effect. As expected, in J82-V, DHT did not increase cell growth, while EGF and EGF+DHT, although less significant, induced it by 17% (p=0.010) and 20% (p=0.043), respectively. Additionally, HF failed to block the EGF effect in J82-V cells. These results suggest that EGF promotes bladder cancer cell proliferation at least partially through the AR pathway.

EGF increases AR and TIF2 expression. To further investigate how EGF influences AR signals, we examined AR expression by western blotting. In UMUC3, AR expression was increased by DHT (4.4-fold) and further enhanced by addition of EGF (6.4-fold), whereas no significant effect of EGF or HF was seen in the absence of DHT (Fig. 4A). HF clearly antagonized the effects of DHT with or without EGF. In J82-AR, EGF appeared to increase AR expression both in the presence (2.8-fold) and
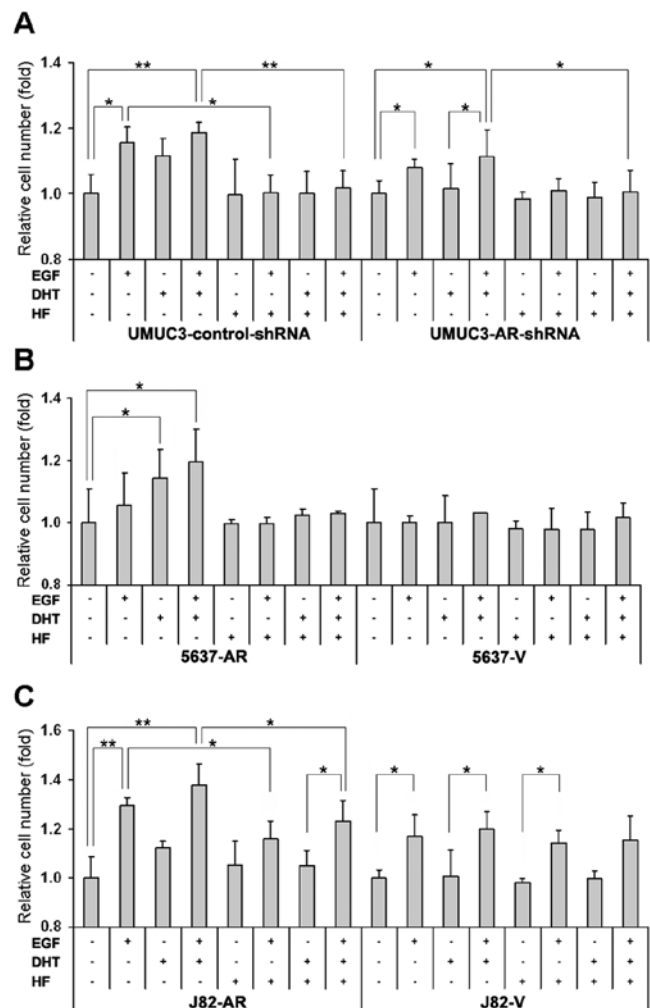
Figure 3. Effects of EGF on cell viability. Bladder cancer cells (A, UMUC3-control-shRNA/AR-shRNA; B, 5637-AR/vector; C, J82-AR/vector) were cultured for 4 days in the presence of ethanol (mock), 100 ng/ml EGF, 10 nM DHT and/or 10 μM HF, as indicated. Cell viability was assayed with MTT and growth induction is presented relative to cell number with mock treatment estimated by measuring the absorbance at a wavelength of 570 nm with a background subtraction at 655 nm (first lanes; set as 1-fold). Each value represents the mean + SD from at least three independent experiments. *p<0.05; **p<0.01.
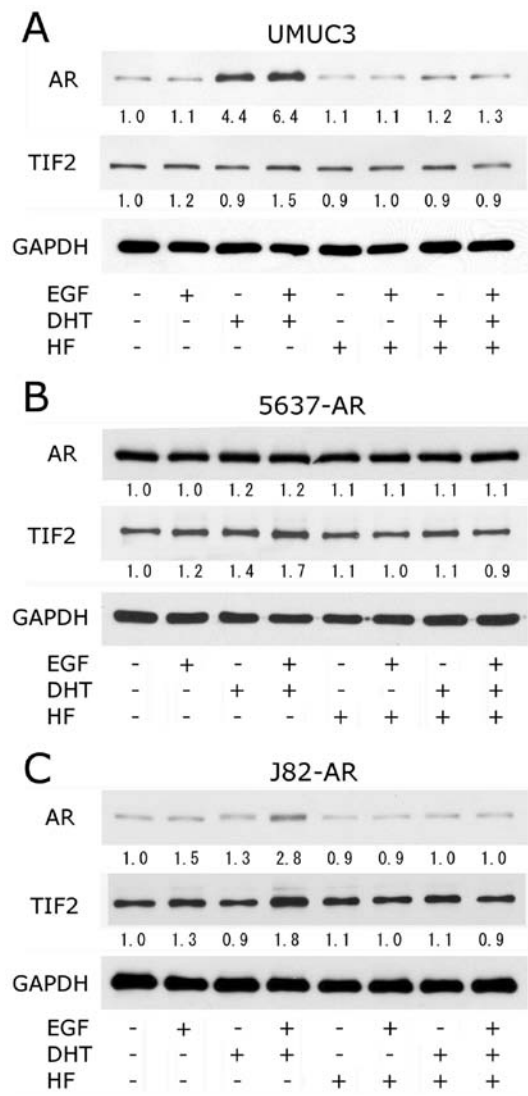

Figure 4. Effects of EGF on AR and TIF2 protein expression. Bladder cancer cells (A, UMUC3; B, 5637-AR; C, J82-AR) were cultured for 24 h in the presence of ethanol (mock), 100 ng/ml EGF, 10 nM DHT and/or 10 μM HF, as indicated. Equal amounts of protein extracted from each cell line were immunoblotted for AR (110 kDa, upper), TIF2 (160 kDa, middle), or GAPDH (37 kDa, lower) as indicated. Densitometry values for specific bands standardized by GAPDH that are relative to those of mock treatment (first lanes; set as 1-fold) are included below the lanes.

absence (1.5-fold) of DHT and HF abolished these effects (Fig. 4C). In contrast, only marginal effects of EGF and/or DHT on AR expression were observed in 5637-AR (Fig. 4B).

Because EGF was shown to induce AR transcription by upregulating TIF2 expression in prostate cancer cells (21), we then determined the levels of TIF2 expression in bladder cancer cell lines upon treatment with EGF, androgen and/or antiandrogen. As shown in middle panels of Fig. 4, EGF increased TIF2 expression in the presence (1.5- to 1.8-fold) and absence (1.2- to 1.3-fold) of DHT. DHT alone increased TIF2 expression only in 5637-AR (1.4-fold) and showed marginal effects in UMUC3 and J82-AR. In addition, HF abrogated EGF- and/or DHT-enhanced TIF2 expression in all these three lines.

EGF induces AR association with ER and Src. Previous studies in prostate and breast cancers demonstrated that EGF induced AR/ER/Src association, resulting in activation of Src signaling (25,28) and that Src signals phosphorylated tyrosine residue
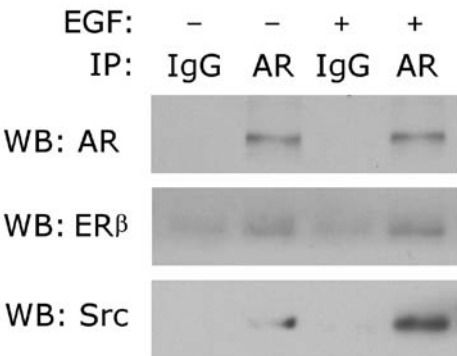

Figure 5. Effects of EGF on AR/ER/Src association. UMUC3 cells were cultured for 24 h in the presence of ethanol (mock) or 100 ng/ml EGF. Cell lysates were immunoprecipitated with anti-AR antibody or normal rabbit IgG and were then immunoblotted for AR (110 kDa), ERβ (56 kDa), or Src (60 kDa), as indicated.

of AR, provoking its transactivation and cell proliferation (29). We therefore investigated whether EGF induced AR/ER/Src complex formation in UMUC3 which is ERα-negative/ERβ-positive (figure not shown). As shown in Fig. 5, both Src and ERβ were co-immunoprecipitated with AR in bladder cancer cells. Furthermore, EGF treatment facilitated the association of AR with ERβ or Src.

Discussion

Dysregulation of the EGFR family is well known to associate with bladder cancer (14-16). AR signals have also been implicated in bladder carcinogenesis and tumor progression (3,5,7,9-13). Nonetheless, crosstalk between the AR and EGFR pathways remains unclear in bladder cancer, although it has been widely studied in prostate cancer (20-24). We have recently shown that AR signals increase EGFR and ERBB2 expression and activity, suggesting androgen-mediated bladder cancer progression via the regulation of the EGFR/ERBB2 pathways (26). In the present study, we provided evidence suggesting that EGF could regulate cell proliferation by activating AR signals in bladder cancer.

In prostate cancer, accumulating evidence has indicated that EGFR/ERBB2 signals induce AR transactivation in an androgen-dependent and -independent manner (20-22). In bladder cancer cells, we here showed that EGF could activate AR transcription and PD168393, a specific inhibitor of EGFR, restored this EGF effect. These data suggest that EGF androgen-independently induces EGFR-mediated ARE reporter activity in bladder cancer. However, it was shown that the effect of EGF on AR transcription might be almost negligible compared to the induction by androgens in prostate cancer (20,21). Similarly, in bladder cancer lines 5637-AR and J82-AR where a wild-type AR was stably overexpressed, the effect of EGF was less significant than that of DHT. On the other hand, in UMUC3 cells that possess endogenous AR, EGF effect (20% increase) is similar to the relatively insignificant effect of DHT (25% increase). In addition, PD168393 displayed agonist effects [1.5-fold (vs. 2.0-fold by EGF or 7.4-fold by DHT)] on AR transcription in J82-AR via unknown mechanisms. It was described in prostate cancer cells that PD168393 upregulated AR target gene expression in the presence of androgen, possibly via blocking basal activity of EGFR or ERBB2 (30). Importantly, as shown in prostate cancer (21), a combination of EGF and androgen further induced AR transcriptional activity in all the three bladder cancer lines tested and the AR antagonist HF completely abolished AR transactivation induced by EGF, androgen, or both at least in UMUC3. We could not evaluate antagonistic effects of HF on EGF-induced AR transcription due to the considerable agonist activity of HF which was even higher than that of EGF in 5637-AR. Thus, our results support the possibility that EGF mediates AR transcriptional activity through the EGFR and AR pathways in bladder cancer cells.

Consistent with previous findings shown by others and us (5,7,9,26) androgens promoted AR-positive bladder cancer cell proliferation that was blocked by antiandrogens. These effects of androgens were suggested to be at least partially mediated through the EGFR pathway (26). In the present study, as expected, EGF increased the growth of AR-positive cells and, less significantly, that of AR-knockdown/negative cells. In AR-positive lines, combined treatment with EGF and androgen further induced cell proliferation. Of note were inhibitory effects of the AR antagonist on EGF- and EGF+androgen-increased cell growth. Specifically, on the growth of 5637-derived lines, EGF and/or DHT showed only marginal effects (5637-V) and HF almost completely abolished EGF-mediated effects (5637-AR). These findings indicate that EGF-induced cell proliferation involves the AR pathway in bladder cancer. Nonetheless, in J82-derived lines, EGF retained its effect on cell growth without AR (J82-V) and HF failed to completely inhibit EGF-increased cell proliferation (J82-AR), suggesting the involvement of those other than the AR pathway.

It has been reported that EGF is capable of inducing AR transcription and protein expression in androgen-independent prostate cancer cells (21). Others also described negative regulation of AR expression and activity by EGFR signaling in prostate cancer (30,31). In bladder cancer cells, we previously showed increases in the expression of endogenous AR by androgen treatment (26), which was inconsistent with the results demonstrated by Boorjian et al (9). We also showed no significant increases in exogenously overexpressed AR (5637-AR) by DHT or in endogenous and exogenous ARs by EGF (26). We confirmed our previous findings in the three lines tested and further showed EGF-enhanced AR overexpression in the presence of androgen in UMUC3 and J82-AR, but not in 5637-AR. The mechanism underlying this discrepancy in the response to the treatment of EGF+DHT between levels of exogenous AR expression in 5637 versus J82 remains uncertain. Repeatedly, the AR expression increased by androgen with or without EGF in bladder cancer cells was abolished by an AR antagonist.

EGF has been shown to enhance the expression or phosphorylation of TIF2, one of the p160 nuclear receptor coactivators, leading to an increase in AR transactivation in prostate cancer cells (21). Indeed, the expression of major AR coactivators, including TIF2, was detected in bladder cancer cell lines as well as in AR-positive and even AR-negative bladder tumor specimens and TIF2 knockdown resulted in a decrease in androgen-mediated cell proliferation (9). We here found that TIF2 was considerably (e.g., ≥1.5-fold) augmented in the presence of EGF and DHT in bladder cancer cells, while EGF or DHT alone could lead to marginal/only slight increases in TIF2 expression. Interestingly, like our results in AR expression/activity and cell proliferation, EGF-induced TIF2 upregulation was abolished by the antiandrogen. Although detailed mechanisms need to be clarified, these results may imply that elevated levels of TIF2 contribute to EGF/androgen-enhanced AR transactivation in bladder cancer cells.

In hormone-responsive cells expressing both AR and ER (α and/or β), such as prostate and breast cancers, AR/ER/Src association plays a crucial role in activation of Src signals triggered by EGF and/or sex hormones (25,28). It was noteworthy that either AR or ER antagonist sufficiently inhibited this EGF-mediated association and subsequent stimulatory effects (28). It has also been shown that Src mediates EGF-induced AR tyrosine phosphorylation in prostate cancer cells, which leads to an increase in AR transcriptional activity (29). Indeed, in many bladder cancer tissue specimens, AR and ER(s) were found to be co-expressed (3,10,12). In this study, we showed associations of AR with ERβ and Src in UMUC3 which were enhanced by EGF treatment. These findings suggest that EGF activates Src via assembling the AR/ER/Src complex, resulting

in AR transactivation and cell proliferation in bladder cancer. This may also justify the drastic inhibition of EGF-induced effects accomplished by antiandrogen treatment.

In conclusion, EGF could increase AR transcriptional activity and cell proliferation in bladder cancer. These EGF effects were likely mediated through the AR pathway involving upregulation of TIF2 expression as well as activation of Src signals due to forming an AR/ER/Src complex. These results, together with our previous findings, not only shed light on crosstalk between the AR and EGFR pathways in bladder cancer but also enhance the feasibility of androgen deprivation interfering with this crosstalk as a potential therapeutic approach.

Acknowledgements

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Therapeutic Discovery

Contrary Regulation of Bladder Cancer Cell Proliferation and Invasion by Dexamethasone-Mediated Glucocorticoid Receptor Signals

Yichun Zheng[1,2], Koji Izumi[1], Yi Li[1,2], Hitoshi Ishiguro[1], and Hiroshi Miyamoto[1]

Abstract

In patients with advanced bladder cancer, glucocorticoids are frequently given to reduce acute toxicity, particularly hyperemesis, during chemotherapy, as well as to improve cachectic conditions. However, it remains unclear whether glucocorticoids directly affect the development and progression of bladder cancer through the glucocorticoid receptor pathway. Glucocorticoid receptor expression was first investigated in human bladder cancer lines and tissue microarrays. Then, the effects of dexamethasone on glucocorticoid receptor transcription, cell proliferation, apoptosis/cell cycle, and invasion were examined in bladder cancer lines. Finally, mouse xenograft models for bladder cancer were used to assess the efficacy of dexamethasone on tumor progression. All the cell lines and tissues examined were found to express glucocorticoid receptor. Dexamethasone increased glucocorticoid receptor–mediated reporter activity and cell proliferation, and inhibited apoptosis in the presence or absence of cisplatin. In contrast, dexamethasone suppressed cell invasion, the expression of its related genes [*MMP-2/MMP-9*, *interleukin (IL)-6*, *VEGF*], and the activity of MMP-2/MMP-9, and also induced mesenchymal-to-epithelial transition. In addition, dexamethasone increased IκBα protein levels and cytosolic accumulation of NF-κB. In xenograft-bearing mice, dexamethasone slightly augmented the growth of the inoculated tumors but completely prevented the development of bloody ascites, suggestive of peritoneal dissemination of tumor cells, and actual metastasis. In all these assays, dexamethasone effects were abolished by a glucocorticoid receptor antagonist or glucocorticoid receptor knockdown via RNA interference. Thus, glucocorticoid receptor activation resulted in promotion of cell proliferation via inhibiting apoptosis yet repression of cell invasion and metastasis. These results may provide a basis of developing improved chemotherapy regimens, including or excluding glucocorticoid receptor agonists/antagonists, for urothelial carcinoma. *Mol Cancer Ther; 11(12); 2621–32. ©2012 AACR.*

Introduction

Dichotomous genetic pathways have been implicated in urothelial carcinoma of the urinary bladder, leading to the development of clinicopathologically distinct types of tumors: low-grade, mostly noninvasive; and high-grade, often invasive (1, 2). Patients with low-grade tumors carry a lifelong risk of frequent (50%–70%) recurrence, occasionally with grade/stage progression, whereas high-grade carcinomas are often life-threatening despite currently available aggressive treatment modalities, including radical cystectomy and systemic chemotherapy in the neoadjuvant, adjuvant, or salvage setting. Therefore, novel therapeutic options that prevent tumor recurrence and/or progression need to be developed.

Glucocorticoids are involved in almost every cellular, molecular, and physiologic network of the organism and represent one of the most commonly prescribed drugs often used in the treatment of inflammatory and autoimmune disorders. Several glucocorticoids have also been clinically used as cytotoxic agents, predominantly for hematologic malignancies (3). Conversely, there are only limited amounts of experimental evidence suggesting that glucocorticoids inhibit cell growth of solid tumors, such as prostate cancer (4). In bladder cancer cells, Zhang and colleagues (5, 6) have shown *in vitro* evidence suggesting glucocorticoid-induced resistance to cytotoxic effects of cis-diamminedichloroplatinum (CDDP), currently the most effective agent against urothelial carcinoma. Nonetheless, in patients with solid tumors, glucocorticoids are frequently given to reduce acute toxicity, particularly hyperemesis during chemotherapy, to protect normal tissue

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against the long-term effects of genotoxic drugs, and to improve cachectic conditions (7). Because of these benefits, a glucocorticoid is often included as comedication in the standard chemotherapy regimens for bladder cancer. Meanwhile, prolonged systemic use of glucocorticoids has been shown to increase the subsequent risk of bladder cancer, possibly due to immunosuppression (8).

Accordingly, it remains unanswered whether glucocorticoids directly affect the development and progression of bladder cancer, presumably through glucocorticoid receptor (GR), a member of the nuclear receptor superfamily that functions as a ligand-inducible transcription factor. Recently, we and others showed that signaling pathways of other steroid hormone receptors, such as androgen receptor (AR) and estrogen receptors, play an important role in bladder cancer progression (9–11). In this study, we aim to determine whether and how glucocorticoid receptor signals regulate the growth of bladder cancer.

Materials and Methods

Cell culture and chemicals

Human urothelial carcinoma cell lines UMUC3, TCC-SUP, 5637, and J82, and human embryonic kidney cell line 293T (all obtained from the American Type Culture Collection) were maintained in appropriate medium (Mediatech; RPMI-1640 for 5637 and Dulbecco's Modified Eagle's Medium for others) supplemented with 10% fetal bovine serum (FBS). Cells were cultured in phenol-red-free medium supplemented with 5% charcoal-stripped FBS (CS-FBS) at least 18 hours before experimental treatment. Although cell lines were not authenticated by the authors, cells were immediately expanded after receipt and stored in liquid nitrogen and were not cultured for more than 5 months following resuscitation. We obtained dexamethasone (DEX; Fig. 1A), mifepristone (RU486), dihydrotestosterone (DHT), CDDP, and TNF-α from Sigma. Supplementary Table S1 lists all pertinent information on primary antibodies.

Stable cell lines with glucocorticoid receptor-short hairpin RNA

To establish stable glucocorticoid receptor knockdown lines, UMUC3 and TCC-SUP were directly infected with GR-short hairpin RNA (shRNA) or control-shRNA lentiviral particles (Santa Cruz Biotechnology) in the presence of 5 μg/mL polybrene (Millipore), as described for AR knockdown (12). After 48 hours of infection, the target cells were selected by 2 μg/mL puromycin (Sigma).

Reporter gene assay

Cells seeded in 24-well plates were cotransfected with 250 ng of mouse mammary tumor virus (MMTV)-luc reporter plasmid DNA and 2.5 ng of PRL-TK-luc plasmid DNA, using GeneJuice (Novagen), as described previously (9, 12), and cultured in medium supplemented with CS-FBS for 24 hours in the presence of dexamethasone and/or RU486. The harvested cells were assayed for luciferase

activity determined using a Dual-Luciferase Reporter Assay kit (Promega) and luminometer (TD-20/20, Turner BioSystems).

Cell proliferation

We used the MTT (thiazolyl blue) assay to assess cell viability, as described previously (9, 12). Briefly, cells (3×10^3) seeded in 96-well plates were incubated with medium supplemented with or without CS-FBS containing ligands (DEX/RU486) and/or CDDP. After 4 days of treatment, we added 10 μL of MTT (Sigma) stock solution (5 mg/mL) to each well with 0.1 mL of medium for 4 hours at 37°C. Then, we measured the absorbance at a wavelength of 570 nm with background subtraction at 655 nm.

Cell morphology

Morphology of cells cultured with dexamethasone and/or RU486 was assessed, using the NIH ImageJ software. Parameters included the area, perimeter, circularity, and roundness.

Transwell assay

Cell invasiveness was determined, using a Matrigel (30 μg; BD Biosciences)-coated transwell chamber (5.0 μm pore size polycarbonate filter with 6.5 mm diameter; Costar). Cells (1×10^5) in 100 μL of serum-free medium were added to the upper chamber of the transwell, whereas 600 μL of medium containing 5% FBS was added to the lower chamber. The media in both chambers contained ligands (DEX/RU486). After incubation for 36 hours at 37°C in a CO_2 incubator, invaded cells were fixed, stained with 0.5% crystal violet, and counted under a light microscope.

Flow cytometry

Cells (1×10^6/10-cm dish) were cultured in medium supplemented with CS-FBS containing ligands (DEX/RU486) for 24 hours, harvested with trypsin, fixed in 70% ethanol, and stained with propidium iodide (PI) buffer. Cellular PI content was measured on a BD FACS-Canto flow cytometer (BD Biosciences) equipped with an argon ion laser at 488 nm wavelength. Data were analyzed using FlowJo software (Tree Star).

Gelatin zymography

Cells (1×10^6 cells/10-cm dish) were cultured in serum-free medium containing ligands (DEX/RU486) at 37°C in a CO_2 incubator for 24 hours. The conditioned medium was collected/centrifuged and electrophoresed in 8% polyacrylamide gels copolymerized with 1 mg/mL gelatin. After washing and overnight incubation at 37°C in a buffer containing 50 mmol/L Tris, 5 mmol/L $CaCl_2$, and 1 μmol/L $ZnCl_2$, the gels were stained with 0.4% Coomassie blue.

Reverse transcription and real-time PCR

Total RNA (0.5 μg) isolated from cultured cells, using TRIzol (Invitrogen), was reverse transcribed using 1

Figure 1. Dexamethasone (DEX) effects on glucocorticoid receptor (GR) transactivation. A, chemical structure of dexamethasone. Bladder cancer cells (B, UMUC3/TCC-SUP/J82/5637; C, UMUC3/TCC-SUP-control-shRNA/GR-shRNA; D, UMUC3-control-shRNA/AR-shRNA) transfected with MMTV-luc were cultured for 24 hours in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 1 μmol/L RU486. Luciferase activity is presented relative to that of mock treatment in each control line. Each value represents the mean (+SD) from at least 3 independent experiments.
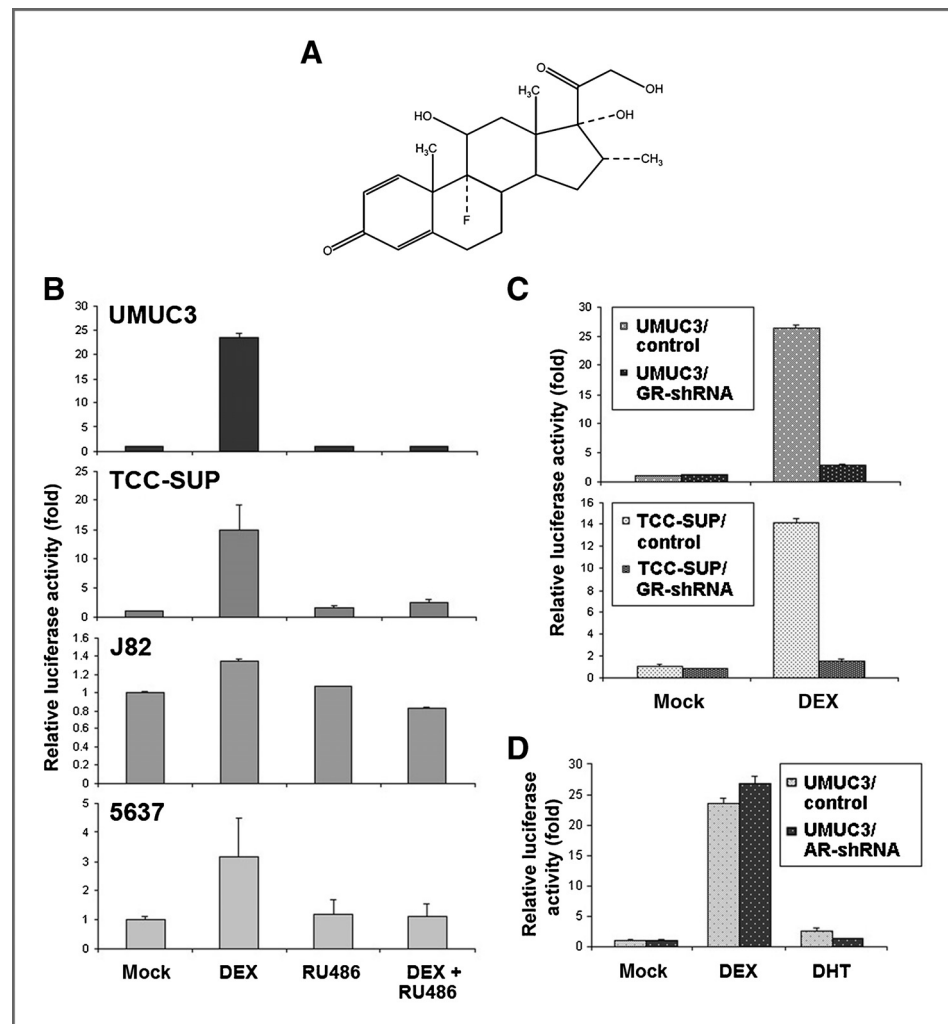
μmol/L oligo (dT) primers and 4 units of Ominiscript reverse transcriptase (Qiagen) in a total volume of 20 μL. Real-time PCR was then carried out, using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously (12). The primer sequences are given in Supplementary Table S2.

Western blot analysis and coimmunoprecipitation

Whole-cell protein extraction and Western blot analyses were conducted, as described previously (12) with minor modifications. Separate cytoplasmic and nuclear protein fractions were obtained, using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Protein (30 μg) was separated in 10% SDS-PAGE and transferred to polyvinylidene diflouride membrane (Millipore). Specific antibody binding was detected, using horseradish peroxidase detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific).

For immunoprecipitation, whole-cell lysates in 500 μL were precleared with 15 mL of protein A/G beads (Santa Cruz Biotechnology) for 30 minutes at 4°C. After centrifuging, supernatant was incubated with an anti-

body overnight at 4°C followed by addition of 25 μL A/G agarose beads for 2 hours. The beads were washed and the proteins were separated by SDS-PAGE for Western blot analysis.

Apoptosis

The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted on cell-burdening coverslips and rehydrated sections from paraffin-embedded mouse xenograft tumors, using the DeadEnd Fluorometric TUNEL System (Promega), followed by counterstaining for DNA with 4′,6-diamidino-2-phenylindole (DAPI). Apoptotic index was determined in the cells visualized by fluorescence microscopy.

Immunofluorescent staining

Cells plated onto 22-mm square coverslips in 6-well plates were cultured in medium with CS-FBS containing dexamethasone and/or TNF-α for 24 hours. Culture medium was then aspirated, and the adherent cells were fixed by 4% paraformaldehyde for 10 minutes at

room temperature. After being washed with 0.1 M glycine for 20 minutes at room temperature, the coverslips were kept in 1% Triton X-100 for 20 minutes at 37°C and blocked with blocking buffer for 1 hour at 37°C. Primary antibodies were incubated at 4°C overnight, and Alexa 488- or 568-conjugated secondary antibodies (Invitrogen) were added for 45 minutes at 37°C. Fluorescence images were acquired with an Olympus FV1000 confocal microscope.

Mouse xenograft models

Bladder cancer lines (1×10^6 cells in 100 μL per site) resuspended in Matrigel (BD Biosciences) were inoculated subcutaneously into the right (GR-shRNA) and left (control-shRNA) flanks of 7-week-old male severe combined immunodeficient (SCID) mouse (NCI). Slow-releasing pellets [dexamethasone (0.5 mg/mouse) or placebo, Innovative Research of America] were injected with a precision trochar when the sizes of all tumors in each group reached 40 mm³. Tumors were measured using calipers and tumor weight was calculated by the following formula: tumor weight (mg) = tumor length (mm) × [tumor width (mm)]² × 0.5 (9). After 5 weeks of treatment, the mice were killed and all the tumors including metastases were harvested for histologic and immunohistochemical assessment.

Bladder tissue microarray and immunohistochemistry

Appropriate approval from the Institutional Review Board of the University of Rochester (Rochester, NY) was obtained before construction and use of the tissue microarray (TMA). Bladder TMA was constructed from 24 cystectomy specimens, as described previously (12, 13). These patients included 19 men and 5 women, with a mean follow-up after the surgery of 11.4 months (range, 3–24). All cases were histologically diagnosed as high-grade urothelial carcinoma, including 5 ≤pT1, 19 ≥pT2, 12 pN0, and 12 pN+ tumors. None of the patients had received radiotherapy or systemic chemotherapy with or without glucocorticoids preoperatively.

Immunohistochemical staining was conducted on the sections (5-μm thick) from the bladder TMA and xenograft tumors, as described previously (9, 12). Briefly, tissues were deparaffinized in xylene, rehydrated in a graded ethanol series, and incubated in 3% hydrogen peroxide. Samples were incubated overnight at 4°C with a primary antibody and then with a broad spectrum secondary antibody (Invitrogen). The stains were manually scored by one pathologist (H. Miyamoto) blinded to sample identity.

Statistical analyses

Differences in variables with a continuous distribution were analyzed by Student t test. Differences in glucocorticoid receptor expression rates in human tissue samples were compared using Fisher exact test. Progression-free survival rates were calculated by the Kaplan–Meier method and comparison was made by log-rank test. $P < 0.05$ was considered to be statistically significant.

Results

Glucocorticoid receptor expression

We first examined the expression of glucocorticoid receptor in 4 human bladder cancer lines, UMUC3, TCC-SUP, J82, and 5637, by reverse transcription PCR (RT-PCR) and Western blot analysis. All the lines were found to express glucocorticoid receptor at both mRNA (Supplementary Fig. S1A) and protein (Supplementary Fig. S1B) levels. Silencing of glucocorticoid receptor expression in UMUC3-GR-shRNA and TCC-SUP-GR-shRNA was then confirmed (see Fig. 2F).

Next, we immunohistochemically stained for glucocorticoid receptor in the bladder TMA. Positive signals were detected typically in both nuclei and cytoplasms of epithelial cells (Supplementary Fig. S1C). All the nonneoplastic and neoplastic bladders as well as metastases showed at least weak signals in urothelial cells. Strong signals were found in 5/24 (21%) primary tumors, 0/4 (0%) metastatic lymph nodes, and 8/18 (44%) corresponding benign tissues. Thus, glucocorticoid receptor expression tended to be weaker in urothelial carcinoma than in benign urothelium ($P = 0.0916$). There were no statistically significant correlations between the intensity of glucocorticoid receptor expression and gender, presence of muscle invasion (≤pT1 vs. ≥pT2), or status of lymph node involvement. Nonetheless, Kaplan–Meier analysis showed a trend to associate between weak or moderate positivity of glucocorticoid receptor and a risk of progression after radical cystectomy ($P = 0.0925$). No tumor progression was seen in all 5 patients with strongly glucocorticoid receptor-positive tumor.

Dexamethasone-enhanced glucocorticoid receptor transactivation

Glucocorticoid receptor-mediated transcriptional activity was determined in the cell extracts with transfection of a luciferase reporter plasmid (MMTV-luc) and treatment of a synthetic glucocorticoid dexamethasone and/or a glucocorticoid receptor antagonist RU486. Dexamethasone increased luciferase activity in UMUC3 (23.6-fold), TCC-SUP (14.9-fold), J82 (1.4-fold), and 5637 (3.1-fold), compared with respective mock treatments (Fig. 1B). RU486 showing marginal agonist activity could block dexamethasone-induced glucocorticoid receptor transcription in these lines. As expected, dexamethasone effects on luciferase activity were significantly diminished in glucocorticoid receptor knockdown lines (Fig. 1C). Similar induction by dexamethasone was obtained in AR knockdown UMUC3 cells in which DHT failed to increase luciferase activity (Fig. 1D), excluding dexamethasone-mediated MMTV-luc activity via AR. Thus, these bladder cancer cell lines likely possess a functional glucocorticoid receptor.

Dexamethasone-mediated cell proliferation and apoptosis

To see if glucocorticoid affects bladder cancer cell proliferation, each line was cultured with dexamethasone and/or RU486 for 4 days, and cell viability was assessed by MTT assay. Dexamethasone increased cell growth in a dose-dependent manner (up to 41%/66% increases in UMUC3/TCC-SUP, respectively), and RU486 at least partially antagonized the dexamethasone effect (Fig. 2A). We also assessed the effects of dexamethasone and/or RU486 on the growth of stable cell lines with or without CDDP (Fig. 2B). In the absence of CDDP, dexamethasone increased the growth of UMUC3-control/TCC-SUP-control to 26% ($P = 0.0032$)/36% ($P = 0.0003$), respectively, compared with mock treatment, and RU486 antagonized the dexamethasone effect. In addition, growth induction by dexamethasone was found to be more significant (all $P < 0.0001$) when cultured in serum-free conditions (31%/202% increase in UMUC3-control/TCC-SUP-control) or with CDDP (48%/44% increase in UMUC3-control/TCC-SUP-control). In glucocorticoid receptor knockdown lines, only marginal effects of dexamethasone and/or RU486 (except TCC-SUP-GR-shRNA with no serum) were observed. These results are consistent with previous findings in few other bladder cancer lines (5) and further suggest that glucocorticoids promote bladder cancer cell proliferation/inhibit an antiproliferative effect of CDDP through the glucocorticoid receptor pathway.

To investigate how dexamethasone stimulates cell proliferation, we conducted flow cytometry and TUNEL assay. Dexamethasone treatment for 24 hours led to significant increases in G_1-phase cell population in control UMUC3 (57%→78%, $P = 0.0139$) and TCC-SUP (46%→53%, $P = 0.0269$) lines, and RU486 abolished the dexamethasone effects (Fig. 2C). Significant reductions (all $P < 0.01$) in the G_1 proportion were observed in UMUC3-GR-shRNA with respective treatments, compared with UMUC3-control-shRNA, but not in TCC-SUP-control-shRNA versus TCC-SUP-GR-shRNA. In UMUC3-GR-shRNA dexamethasone still increased G_1 fraction from 39% to 51% ($P = 0.0140$), which was blocked by RU486. Thus, dexamethasone appears to induce bladder cancer cell-cycle arrest at G_1 phase. The effects of dexamethasone on apoptosis were then assessed in these lines cultured with or without FBS and CDDP for 4 days (Fig. 2D). In control UMUC3 and TCC-SUP lines with/without FBS, dexamethasone decreased apoptotic indices by 53%/67% and 30%/67%, respectively, and RU486 blocked dexamethasone-induced apoptosis. CDDP (with FBS) significantly increased the index in control UMUC3 (5%→19%; $P = 0.0052$) or TCC-SUP (8%→37%; $P < 0.0001$), and dexamethasone diminished CDDP-induced apoptosis to the levels with mock (+ FBS) treatment (6%/7% in UMUC3/TCC-SUP, $P = 0.0050/P = 0.0001$). RU486 notably increased dexamethasone-inhibited apoptosis in control lines cultured with CDDP. Dexamethasone slightly/significantly reduced CDDP-induced apoptosis in

UMUC3-GR-shRNA (22%→17%, $P = 0.0572$)/TCC-SUP-GR-shRNA (31%→25%, $P = 0.0239$). Thus, dexamethasone-mediated glucocorticoid receptor signals likely prevent apoptosis of bladder cancer cells in the presence or absence of CDDP.

To further investigate the molecular mechanisms of DEX/GR-induced cell proliferation, we conducted Western blot analysis for detecting the expression of cell-cycle- and apoptosis-related molecules (Fig. 2E). No significant changes in the expression of cyclins (D1/D2/D3) and cyclin-dependent kinases (2/4/6) were seen in bladder cancer lines cultured with dexamethasone and/or RU486. However, dexamethasone upregulated the expression of p27 and p21 and downregulated that of cleaved caspase-3 in a dose-dependent manner, and RU486 blocked the dexamethasone effect. The effects of dexamethasone and RU486 on the expression of p21, p27, and cleaved caspase-3 were modest or marginal in glucocorticoid receptor knockdown lines (Fig. 2F). In addition, dexamethasone significantly decreased the levels of glucocorticoid receptor expression.

Dexamethasone-suppressed cell invasion

The effects of glucocorticoid on the invasiveness of bladder cancer cells were assessed, using a transwell invasion assay. Dexamethasone treatment resulted in significant decreases (50%–52%) in the invasive properties of both control lines, and RU486 clearly abolished the dexamethasone effect (Fig. 3A). In glucocorticoid receptor knockdown lines, dexamethasone did not show significant suppressive effects on cell invasion. These data suggest that glucocorticoids inhibit bladder cancer cell invasion through the glucocorticoid receptor pathway.

Using real-time RT-PCR, we then assessed the effects of glucocorticoid/glucocorticoid receptor on the expression of the molecules that play a key role in tumor invasion. Consistent with the results of the transwell assay, dexamethasone decreased the levels of *MMP-2, MMP-9, interleukin (IL)-6,* and *VEGF* by 46%/43%, 47%/66%, 33%/61%, 30%/48% in control UMUC3/TCC-SUP lines, respectively (Fig. 3B). In RU486-treated control and glucocorticoid receptor knockdown lines, inhibitory effects of dexamethasone on the expression of these 4 genes were not significant. MMP-2 and MMP-9 expression was also measured by gelatin zymography to assess their enzymatic activity. Dexamethasone reduced their levels in control lines, but not in control lines cultured with RU486 or in GR-shRNA lines (Fig. 3C).

Dexamethasone-induced mesenchymal-to-epithelial transition

We perceived that dexamethasone-treated bladder cancer cells appeared to be larger and rounder than mock-treated cells (Fig. 4A). Using the ImageJ software, the area, perimeter, circularity, and roundness of the cells were compared among different treatments. Dexamethasone increased these parameters in glucocorticoid receptor–positive lines, compared with those in mock-treated lines,
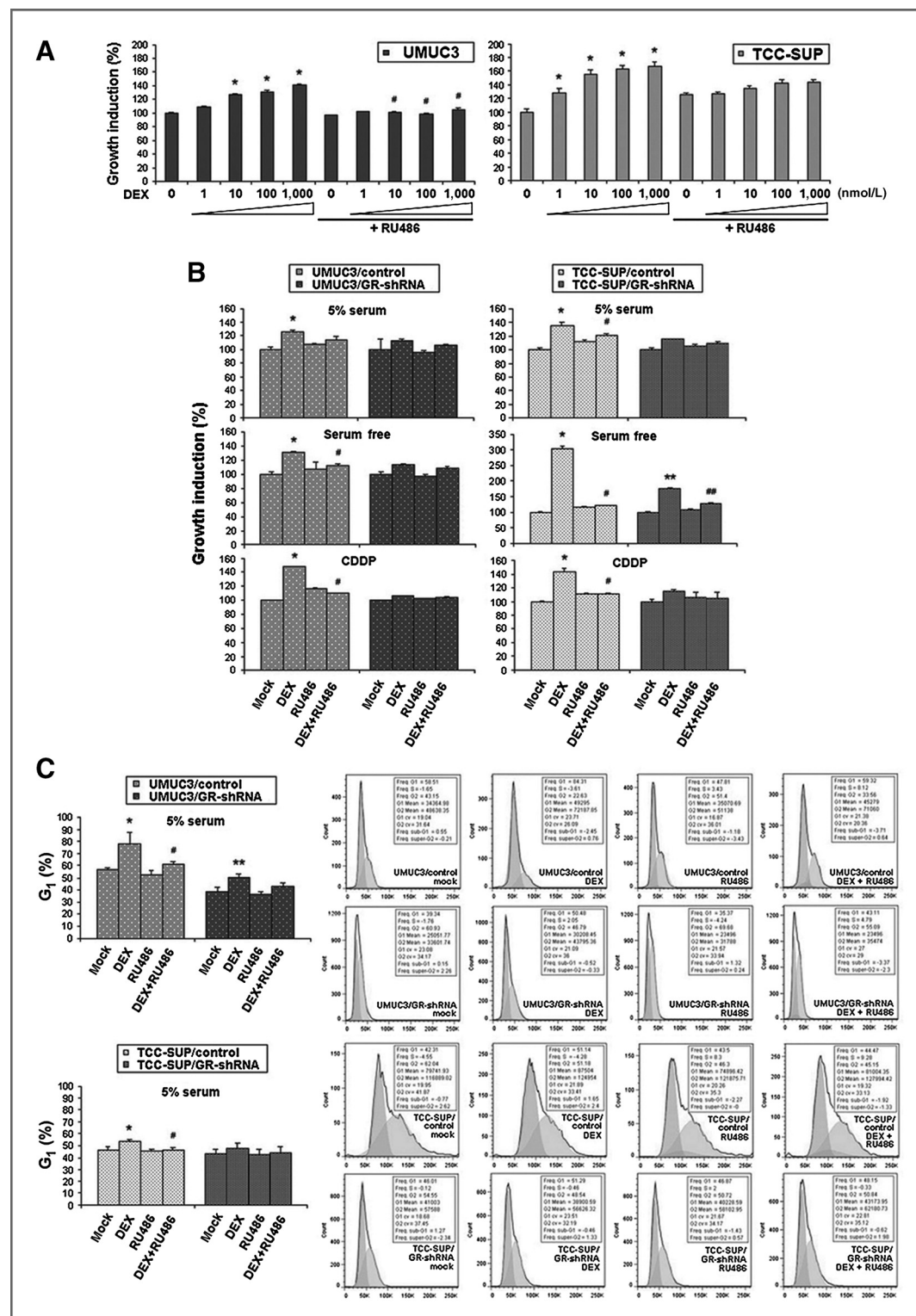
Figure 2. Dexamethasone (DEX) effects on cell proliferation. A, cell viability of UMUC3/TCC-SUP cultured with 0 to 1,000 nmol/L dexamethasone $\pm$ 1 μmol/L RU486 for 4 days was assayed with MTT, and growth induction is presented relative to cell number with mock treatment. Each value represents the mean (+SD) from at least 3 independent experiments. *, $P < 0.05$ (vs. mock treatment); #, $P < 0.05$ (vs. respective doses of dexamethasone treatment only). B, cell viability of UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 5% FBS, no serum, or CDDP (7 μmol/L, with 5% FBS) in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 1 μmol/L RU486 for 4 days was assayed with MTT, and growth induction is presented relative to cell number with mock treatment in each cell line/condition (serum or CDDP). Each value represents the mean (+SD) from at least 3 independent experiments. *, **, $P < 0.05$ (vs. mock treatment); #, ##, $P < 0.05$ (vs. dexamethasone treatment only). C, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 5% FBS in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 1 μmol/L RU486 for 24 hours were used for flow cytometry analysis. Each value for G_1 arrest represents the mean (+SD) from at least 3 independent experiments. *, **, $P < 0.05$ (vs. mock treatment); #, $P < 0.05$ (vs. dexamethasone treatment only). GR, glucocorticoid receptor.
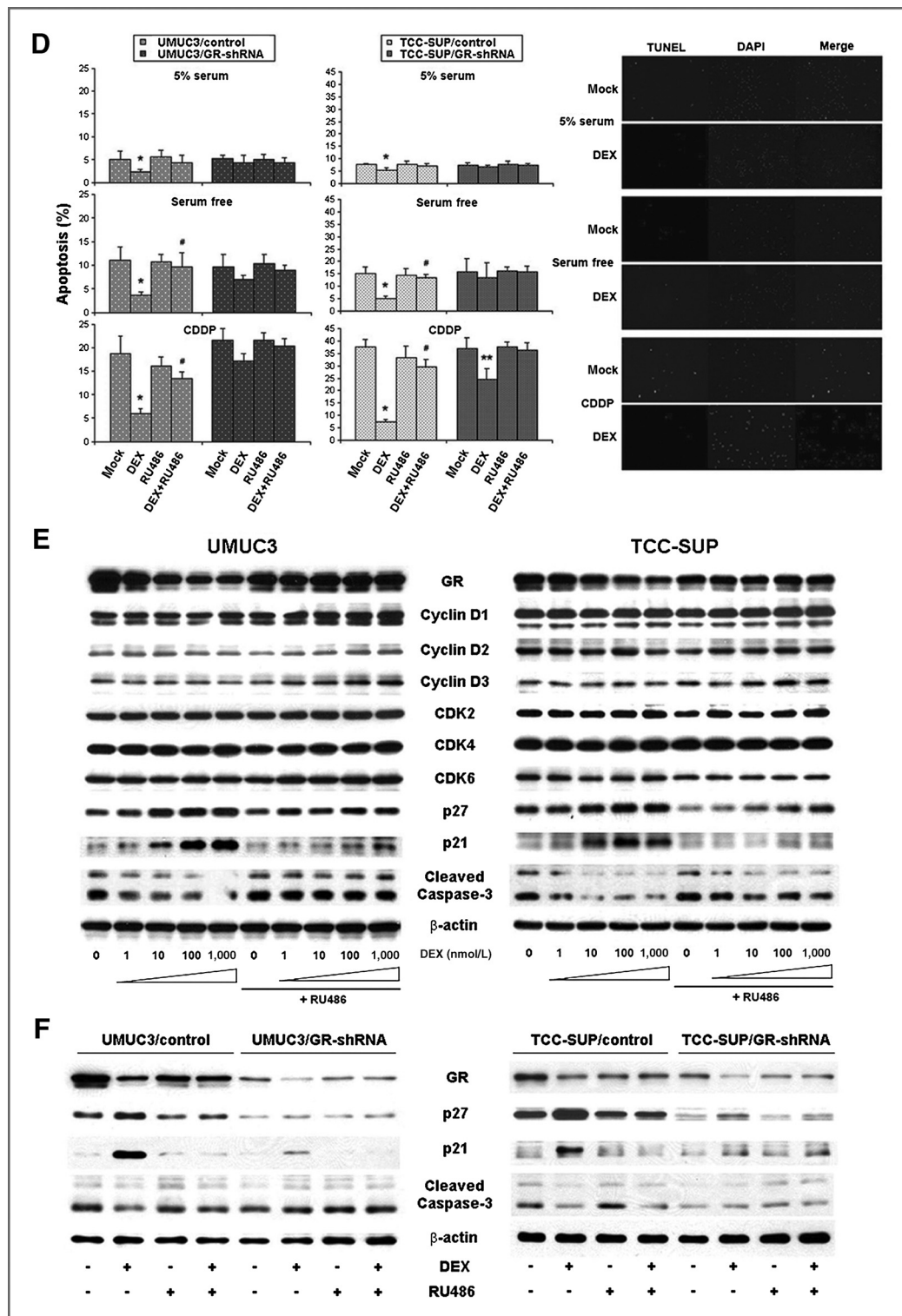
Figure 2. (*Continued*) D, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 5% FBS, no serum, or CDDP (7 µmol/L, with 5% FBS) for 4 days in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 1 µmol/L RU486 were analyzed for apoptotic index (percentage of TUNEL-positive cells in 1,000 cells). Each value represents the mean ($+$SD) from at least 3 independent experiments. *, **, $P < 0.05$ (vs. mock treatment); #, $P < 0.05$ (vs. dexamethasone treatment only). E, UMUC3/TCC-SUP cultured with 0 to 1,000 nmol/L dexamethasone $\pm$ 1 µmol/L RU486 for 24 hours were analyzed on Western blot analysis using an antibody to glucocorticoid receptor (95$+$90 kDa), cyclin D1 (36 kDa), cyclin D2 (31 kDa), cyclin D3 (31 kDa), CDK2 (33 kDa), CDK4 (30 kDa), CDK6 (36 kDa), p27 (27 kDa), p21 (21 kDa), or cleaved caspase-3 (19$+$17 kDa). β-Actin (43 kDa) served as an internal control. F, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured for 24 hours in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 1 µmol/L RU486 were analyzed on Western blot analysis, using an antibody to glucocorticoid receptor, p27, p21, cleaved caspase-3, or β-actin. GR, glucocorticoid receptor.

and RU486 antagonized the dexamethasone effects (Fig. 4A).

To link these results to mesenchymal-to-epithelial transition (MET), we assessed expression levels of mesenchymal (e.g., N-cadherin, vimentin, snail) and epithelial (e.g., E-cadherin, β-catenin) markers by Western blot analysis. In glucocorticoid receptor–positive lines, dexamethasone up-/downregulated the expression of epithelial/mesenchymal markers, respectively, compared with mock treatment (Fig. 4B). These dexamethasone-mediated changes in their levels were marginal or less significant in cells with RU486 treatment and/or glucocorticoid receptor silencing. In addition, basal levels of these epithelial markers were lower in GR knockdown lines than in GR-positive controls.

Dexamethasone-induced disruption of NF-κB

In prostate cancer, dexamethasone could reduce glucocorticoid receptor–positive cell growth via inhibiting NF-κB activation (4, 14). We therefore studied the effects of dexamethasone on NF-κB in bladder cancer cells. Western blot analysis showed increases in the level of IκBα, a natural cytoplasmic inhibitor of NF-κB, but not in NF-κB levels, in both of dexamethasone-treated glucocorticoid receptor–positive lines (Fig. 5A). There were no significant increases in IκBα levels by dexamethasone in cells with RU486 and/or GR-shRNA.

We then conducted coimmunoprecipitation to test if dexamethasone affects protein–protein interactions (Fig. 5B). Dexamethasone induced the interaction between NF-κB and IκBα in control UMUC3 cells, and RU486 or GR-shRNA diminished the dexamethasone effect. Interestingly, glucocorticoid receptor was also pulled down by NF-κB, but not by IκBα.

To further assess whether increased IκBα prevented nuclear translocation of NF-κB, we examined subcellular localization of NF-κB in TCC-SUP by Western blot analysis (Fig. 5C) and immunofluorescence (Fig. 5D). NF-κB localized predominantly to the cytoplasmic compartment in both mock- and dexamethasone-treated cells. Dexamethasone reduced nuclear NF-κB expression, which was prevented by RU486. In particular, dexamethasone blocked nuclear translocation of NF-κB induced by TNF-α. Glucocorticoid receptor localized to the cytoplasm of mock-treated cells and translocated to the nucleus in dexamethasone-treated cells. In TCC-SUP-GR-shRNA, no significant effects of dexamethasone and/or RU486 on subcellular localization of NF-κB were seen (figure not shown).

Dexamethasone increased tumor size but inhibited invasion/metastasis in mouse xenograft models

Finally, we used mouse xenograft models to investigate whether glucocorticoid regulates bladder tumor growth
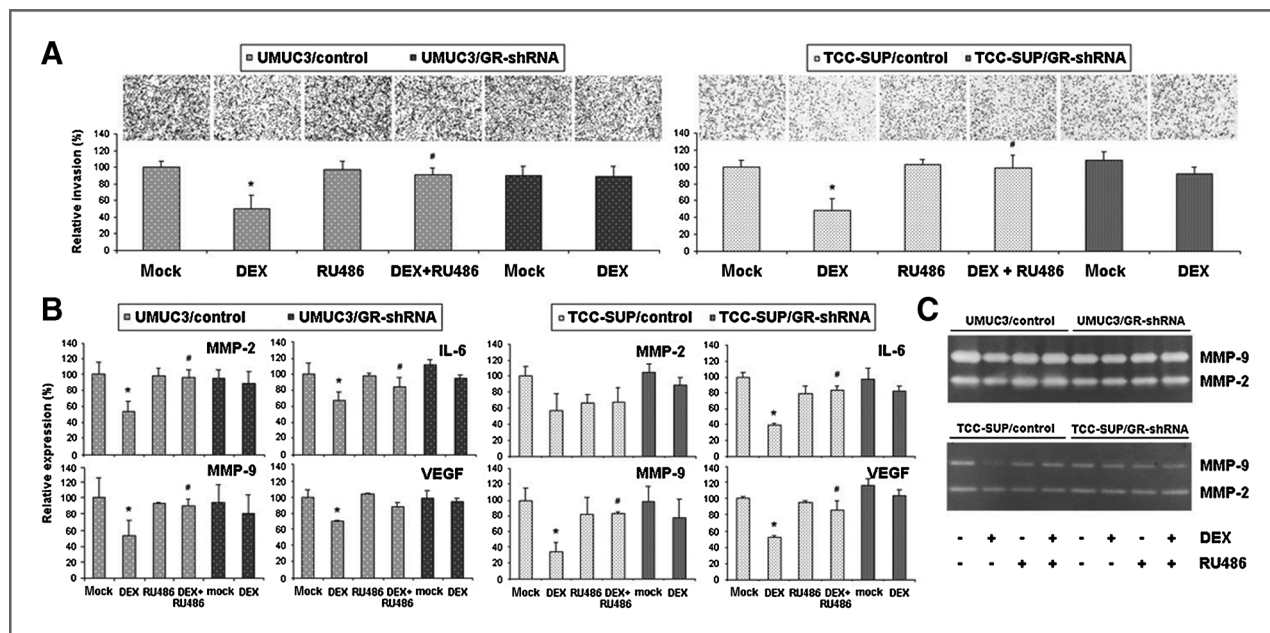

Figure 3. Dexamethasone (DEX) effects on cell invasion. A, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured in the Matrigel-coated transwell chamber for 36 hours in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 1 μmol/L RU486 were used for invasion assay. The number of invaded cells in five random fields was counted under a light microscope (×10 objective). Each value represents the mean (+SD) from 3 independent experiments. *, $P < 0.01$ (vs. mock treatment); #, $P < 0.01$ (vs. dexamethasone treatment only). B, real-time RT-PCR for *MMP-2, MMP-9, IL-6,* and *VEGF* were carried out in UMUC3/TCC-SUP-control-shRNA/GR-shRNA treated with ethanol (mock), 100 nmol/L dexamethasone, and/or 1 μmol/L RU486 for 24 hours. Expression of each specific gene was normalized to that of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Transcription amount is presented relative to that of mock treatment in each cell line. Each value represents the mean (+SD) from at least 3 independent experiments. *, $P < 0.05$ (vs. mock treatment); #, $P < 0.05$ (vs. dexamethasone treatment only). C, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 100 nmol/L dexamethasone and/or 1 μmol/L RU486 for 24 hours were analyzed by SDS-PAGE gelatin zymography for MMP-2 (72 kDa) and MMP-9 (92 kDa). GR, glucocorticoid receptor.

Figure 4. Dexamethasone (DEX) effects on MET. A, UMUC3/TCC-SUP cultured with 100 nmol/L dexamethasone and/or 1 μmol/L RU486 for 24 hours were assessed for cell morphology (area, perimeter, circularity, roundness), using the ImageJ software. Each value represents the mean (+SD) from data in at least 50 cells. *, $P < 0.05$ (vs. mock treatment); #, $P < 0.05$ (vs. dexamethasone treatment only). B, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 100 nmol/L dexamethasone and/or 1 μmol/L RU486 for 24 hours were analyzed on Western blot analysis using an antibody to E-cadherin (128 kDa), N-cadherin (130 kDa), β-catenin (92 kDa), vimentin (57 kDa), or snail (29 kDa). Tubulin (55 kDa) served as an internal control. GR, glucocorticoid receptor.
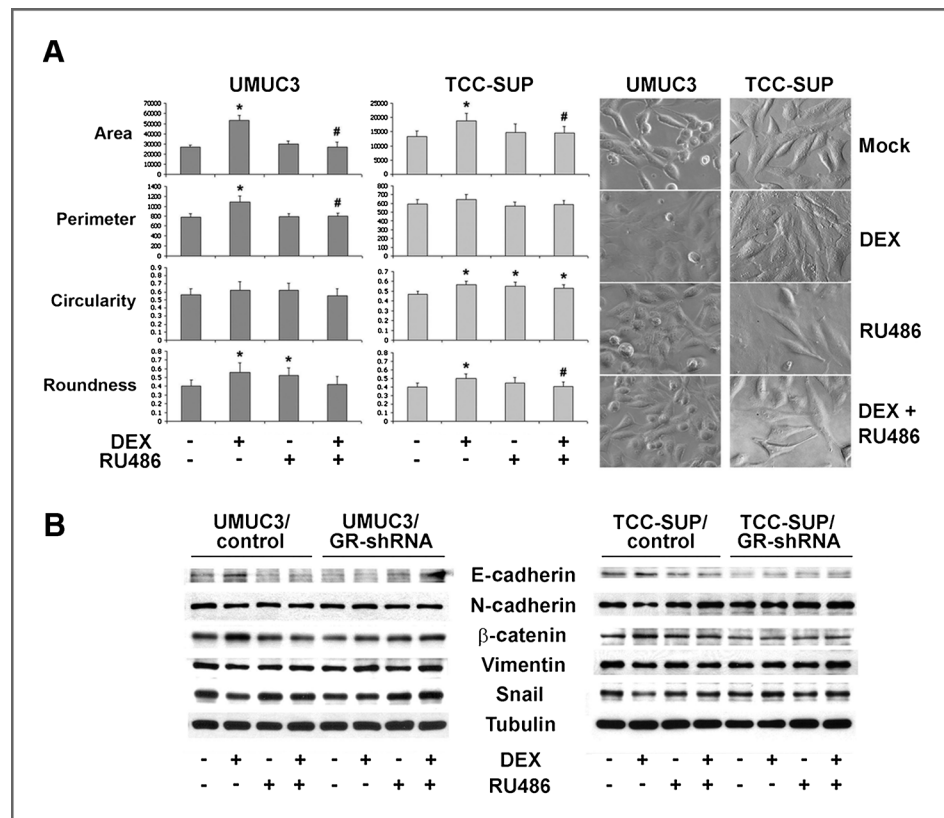
in vivo. Bladder cancer cells (UMUC3-GR-shRNA/control-shRNA) were implanted subcutaneously into the flanks of SCID mice, and after 2 weeks slow-releasing dexamethasone or placebo pellets were injected into mice. Control glucocorticoid receptor–positive tumors in dexamethasone-treated mice were larger/heavier than other tumors at 5 weeks of treatment [e.g., 20%/23% (vs. placebo-control cells); Fig. 6A]. Similarly, placebo/glucocorticoid receptor knockdown tumors were slightly (10%) lighter than placebo/control glucocorticoid receptor–positive tumors, and DEX/GR knockdown tumors were slightly (9%) heavier than placebo/GR knockdown tumors. When the mice were killed, bloody ascites, suggestive of peritoneal dissemination of the tumors, and actual metastatic tumors in the peritoneum were identified in 7 (88%) and 4 (50%) of 8 placebo-treated mice, respectively, but in none of dexamethasone-treated mice. Histologic examination of the tumors revealed invasion into the skeletal muscle in all groups of mice except the control-shRNA/DEX group (Fig. 6B). Harvested tumor specimens were also assessed for cell proliferation (Ki-67), apoptosis (TUNEL), and angiogenesis or metastatic ability (MMP-9/VEGF/CD34). Dexamethasone treatment in control glucocorticoid receptor–positive tumors led to marginal changes in proliferation but decreased apoptosis and angiogenesis/metastasis-related factors, compared with DEX/GR-shRNA, placebo/control-shRNA, placebo/GR-shRNA, or placebo/metastasis (Fig. 6C). Metastatic tumors seen in placebo-treated mice were likely derived from GR-shRNA expressing cells based on weak glucocorticoid receptor signals. It was also noted that glucocorticoid receptor levels were significantly reduced in dexamethasone-treated UMUC3-control-shRNA tumors. These *in vivo* data suggest that dexamethasone stimulates bladder cancer cell proliferation yet represses tumor invasion and metastasis.

Discussion

It appears that the status of glucocorticoid receptor expression has never been examined in human bladder cancer (11). Our immunohistochemical study in 24 cystectomy specimens showed that: (i) glucocorticoid receptor was detected in all cases of benign urothelium/urothelial carcinoma; (ii) glucocorticoid receptor expression tended to be weaker in tumor than in benign; and (iii) strong glucocorticoid receptor expression tended to correlate with better prognosis. These results may suggest a protective/inhibitory role of glucocorticoid receptor signals in bladder tumorigenesis and tumor progression. Further study including larger patient cohorts with longer follow-up are needed to validate these preliminary findings.

It has been shown that glucocorticoids induce apoptosis and inhibit proliferation in lymphoid cells, leading to their clinical use as cytotoxic agents for hematologic malignancies (3). In contrast, limited amounts of experimental evidence have suggested inhibitory effects of glucocorticoids on cell growth of solid tumors. In a previous *in vitro*
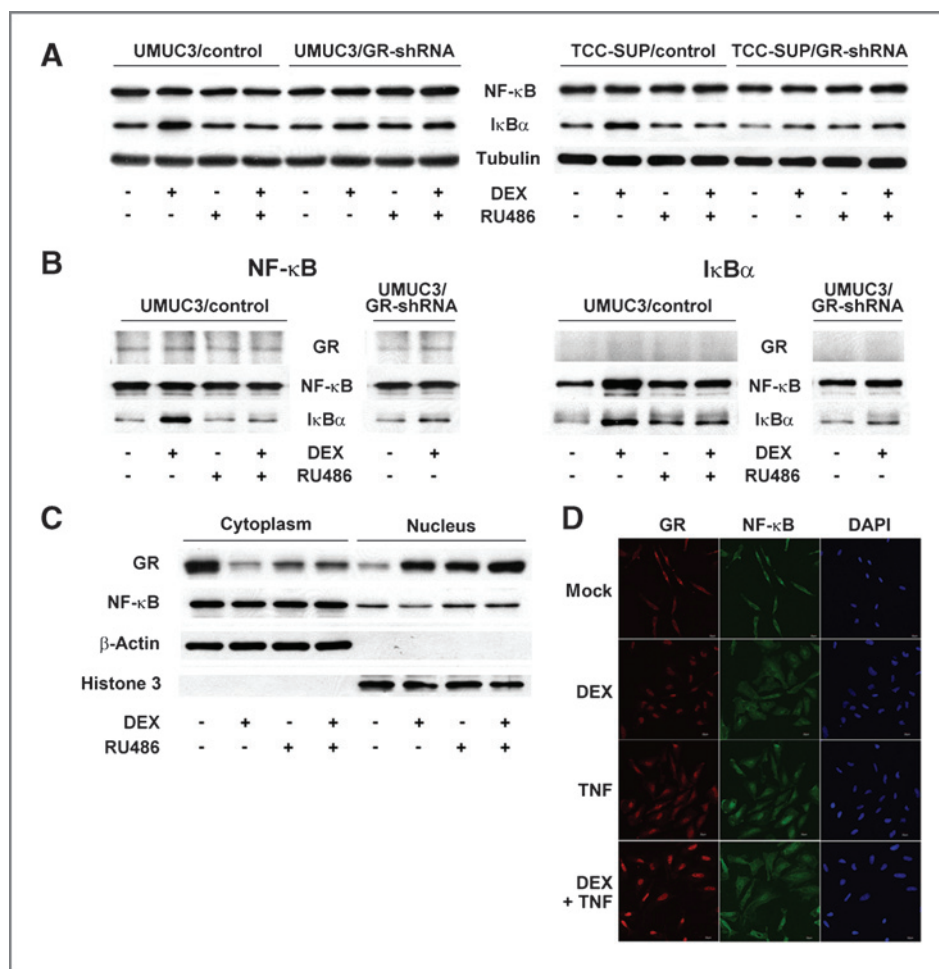
Figure 5. Dexamethasone (DEX) effects on NF-κB. A, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 100 nmol/L dexamethasone and/or 1 μmol/L RU486 for 24 hours were analyzed on Western blot analysis using an antibody to NF-κB (65 kDa) or IκBα (37 kDa). Tubulin (55 kDa) served as an internal control. B, UMUC3-control-shRNA/GR-shRNA were cultured with 100 nmol/L dexamethasone and/or 1 μmol/L RU486 for 24 hours. Cell lysates were immunoprecipitated with anti-NF-κB or IκBα antibody and then immunoblotted for glucocorticoid receptor, NF-κB, or IκBα. C, TCC-SUP was cultured with 100 nmol/L dexamethasone and/or 1 μmol/L RU486 for 24 hours. Separate cytoplasmic and nuclear protein fractions were analyzed on Western blot analysis, using an antibody to glucocorticoid receptor or NF-κB. β-Actin (43 kDa) and histone 3 (15 kDa) served as internal controls for cytoplasmic and nuclear proteins, respectively. D, TCC-SUP cultured for 24 hours in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 20 ng/mL TNF-α were analyzed on immunofluorescence, using an antibody to glucocorticoid receptor or NF-κB. DAPI was used to visualize nuclei. GR, glucocorticoid receptor.

study using bladder cancer lines (5), dexamethasone inhibited CDDP-mediated apoptosis, suggesting glucocorticoid-induced chemotherapy resistance. Using 2 bladder cancer lines expressing a functional glucocorticoid receptor, we here show that dexamethasone promoted cell proliferation, which was restored by a glucocorticoid receptor antagonist and/or glucocorticoid receptor knockdown. The stimulatory effects of dexamethasone were more significant when cultured with CDDP or in serum-free conditions. In addition, dexamethasone-induced cell growth was confirmed, using mouse xenograft models that showed larger tumor sizes in dexamethasone-treated mice than in mock-treated and/or glucocorticoid receptor knockdown cell-bearing mice. Thus, glucocorticoid receptor signals are likely associated with bladder cancer cell growth. Previous (5) and our current results may therefore imply that clinical use of glucocorticoids as comedication can be harmful to patients with bladder cancer in terms of tumor cell proliferation.

Antiproliferative effects of glucocorticoids via induction of cell-cycle arrest and apoptosis have been shown in lymphomas as well as in other malignancies including osteosarcoma, cervical carcinoma, and thyroid medullary carcinoma (3, 15–17). Similarly, dexamethasone enhanced cell cycle at the G_1 phase in 2 glucocorticoid receptor–positive bladder cancer lines, which correlated with increased levels of the CDK inhibitors p27 and p21 but not cyclins (D1/D2/D3) or CDKs (2/4/6). Nonetheless, we found glucocorticoid/glucocorticoid receptor–induced bladder cancer cell proliferation *in vitro* and *in vivo*. In contrast to previous observations in nonbladder cells, dexamethasone strongly inhibited apoptotic cell death, along with downregulation of cleaved caspase-3 expression, in bladder cancer lines and prevented CDDP-induced apoptosis. Because cell line-specific mechanisms for glucocorticoid receptor–mediated growth arrest have been shown (15), further analyses are required to elucidate the involvement of cell-cycle regulatory proteins in bladder cancer cells.

To our knowledge, no studies have assessed the effects of glucocorticoids on bladder cancer cell invasion. Using a transwell assay, we showed that dexamethasone suppressed cell invasion of glucocorticoid receptor–positive lines, but not glucocorticoid receptor knockdown lines, and that RU486 abolished the dexamethasone-induced invasion. In mouse xenograft models for bladder cancer, dexamethasone successfully prevented the development of metastasis. Furthermore, invasion/metastasis-related
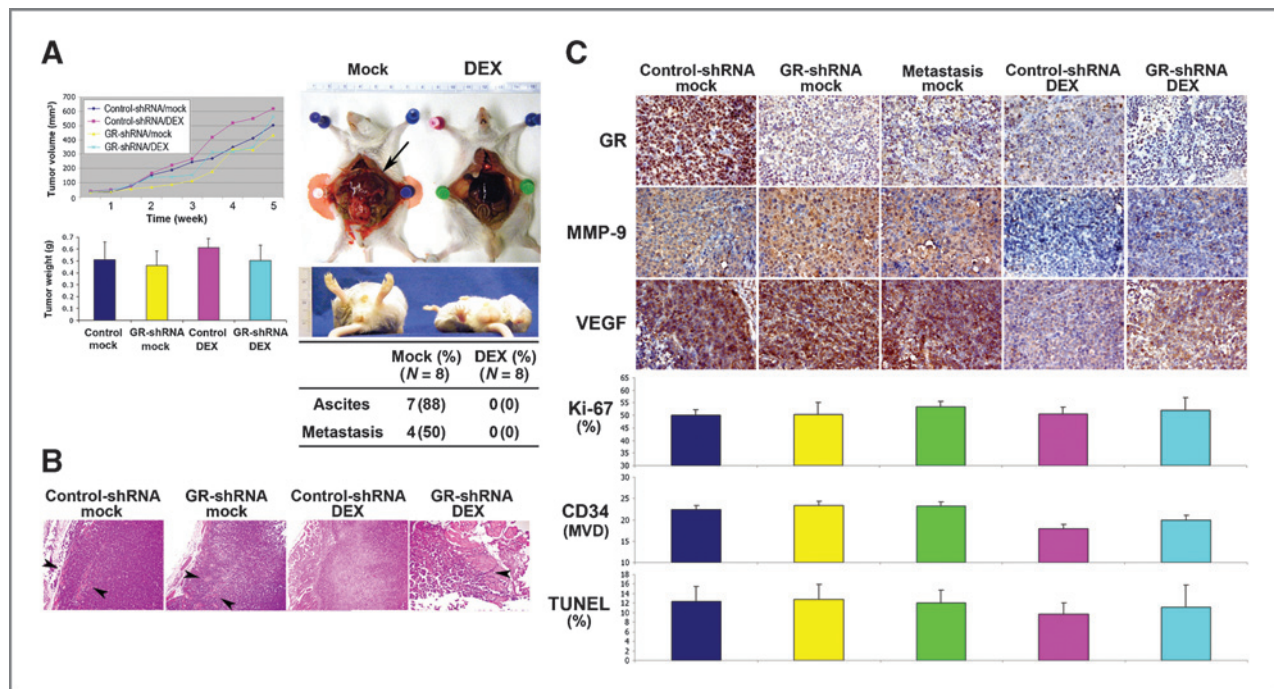
Figure 6. Dexamethasone (DEX) effects on tumor progression in mouse xenograft models. A, UMUC3-GR-shRNA/control-shRNA were implanted subcutaneously into the right/left flanks of male SCID mice, and treatment (injection of dexamethasone or placebo pellet) began when estimated tumor volume reached 40 mm³. Tumor volume ($n = 8$ tumors in each group) was monitored for 5 weeks (left top, mean values). The tumors were then harvested and weighed [left bottom, mean (+SD)]. Bloody ascites and metastatic tumor (arrow) are seen in mock-treated mice (right). B, histology (hematoxylin and eosin staining) of harvested xenograft tumors. Tumor invasion into the skeletal muscle (arrowheads) is seen in all groups of mice except the control-shRNA/DEX group. C, GR/MMP-9/VEGF/Ki-67/CD34 immunohistochemistry and TUNEL analysis in harvested tumors. Mean (+SD) of the percentage of Ki-67–positive cells, microvessel density (MVD; number of vessels highlighted by CD34 staining/high-power field), and the percentage of TUNEL-positive cells are shown. GR, glucocorticoid receptor.

molecules, including MMP-2, MMP-9, IL-6, and VEGF, as well as microvessel density, were downregulated in dexamethasone-treated cells/tumors, compared with mock-treated and/or glucocorticoid receptor knockdown cells/tumors. Thus, opposite to the effect on cell proliferation, glucocorticoids likely have an inhibitory role in bladder cancer cell invasion and metastasis through the glucocorticoid receptor pathway.

Glucocorticoids are known to interfere with the transcriptional activity of several transcription factors, including NF-κB. Dexamethasone has also been shown to reduce the growth of glucocorticoid receptor–positive prostate cancer cells mainly via inhibiting NF-κB activation and the production of NF-κB-dependent cytokines such as IL-6 (4, 14). In bladder cancer, the association of NF-κB activity with cell invasion, as well as the transcriptional regulation of matrix metalloproteinases through the NF-κB pathway, has been reported (18). We therefore assessed the effect of dexamethasone on NF-κB in bladder cancer and found an increase in IκBα level, but not in NF-κB level, in dexamethasone-treated glucocorticoid receptor–positive cells as well as blockade of TNF-α-induced nuclear translocation of NF-κB by dexamethasone. Co-immunoprecipitation further showed dexamethasone-enhanced interactions between NF-κB and IκBα and between glucocorticoid receptor and NF-κB but not IκBα. The latter suggests that glucocorticoid receptor may directly function as a corepressor of NF-κB. Thus, NF-κB inactivation and IL-6 downregulation induced by dexamethasone may be a central mechanism involved in glucocorticoid receptor–mediated inhibition of bladder cancer cell invasion.

During our preliminary experiments, we found changes in the morphology of dexamethasone-treated bladder cancer cells. These might imply dexamethasone-induced MET, which was reported in a mink lung epithelial cell line (19). Indeed, epithelial-to-mesenchymal transition has been implicated in drug resistance and invasion/metastasis in urothelial carcinoma (20). In addition to morphologic changes compatible with MET, we showed that glucocorticoid receptor activation correlated with increased/decreased expression of epithelial/mesenchymal markers, respectively. These findings indicate that DEX/GR induce MET in bladder cancer cells, which could be an underlying mechanism of glucocorticoid receptor–mediated suppression of tumor progression.

As mentioned earlier, glucocorticoids have been widely used as comedication in patients with advanced bladder cancer. However, there was no molecular evidence indicating that glucocorticoids function directly through the glucocorticoid receptor pathway in bladder cancer cells and exert a stimulatory or inhibitory effect on tumor growth. The current study in bladder cancer has shown

that glucocorticoid receptor signals correlate positively with cell proliferation and negatively with cell invasion and metastasis. The former appeared to result in a significant reduction in cytotoxic effects of CDDP. Further analyses using various glucocorticoid receptor agonists/antagonists will facilitate improving chemotherapy regimens for urothelial carcinoma. On the basis of the current results, ideal glucocorticoid receptor ligands would be those showing marginal stimulatory effects on cell proliferation without reducing the cytotoxic activity of anticancer drugs yet significant inhibitory effects on cell invasion either alone or in combination with other agents. Additional assessments of the relationship between glucocorticoid use and tumor phenotype in clinical samples are also required to directly address the hypothesis we tested in cell line models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Zheng, H. Miyamoto
Development of methodology: Y. Zheng, H. Miyamoto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zheng, K. Izumi, Y. Li
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zheng, K. Izumi, Y. Li, H. Ishiguro
Writing, review, and/or revision of the manuscript: Y. Zheng, K. Izumi, Y. Li, H. Ishiguro, H. Miyamoto
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zheng
Study supervision: H. Miyamoto

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Androgen Receptor Signals Regulate UDP-Glucuronosyltransferases in the Urinary Bladder: A Potential Mechanism of Androgen-Induced Bladder Carcinogenesis

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UDP-glucuronosyltransferases (UGTs), major phase II drug metabolism enzymes, play an important role in urinary bladder cancer initiation by detoxifying carcinogens. We aimed to determine if androgens regulate UGT expression via the androgen receptor (AR) pathway in the bladder. Real-time reverse transcription-polymerase chain reaction and Western blot analyses were used to assess UGT1A levels in the normal urothelium SVHUC cell line stably expressed with AR and in bladder tissues from AR knockout (ARKO) and castrated male mice. Immunohistochemistry was also performed in radical cystectomy specimens. Dihydrotestosterone (DHT) treatment in SVHUC-AR reduced mRNA expression of all the UGT1A subtypes (19–75% decrease), and hydroxyflutamide antagonized the DHT effects. In contrast, DHT showed only marginal effects on UGT1A expression in SVHUC-Vector. Of note were higher expression levels of UGT1As in SVHUC-Vector than in SVHUC-AR. In ARKO mice, all the Ugt1a subtypes were up-regulated, compared to wild-type littermates. In wild-type male mice, castration increased the expression of Ugt1a8, Ugt1a9, and Ugt1a10. Additionally, wild-type female mice had higher levels of Ugt1a than wild-type males. Immunohistochemical studies showed strong (3+) UGT1A staining in 11/24 (46%) cancer tissues, which was significantly lower than in corresponding benign tissues [17/18 (94%) cases ($P = 0.0009$)]. These results suggest that androgen-mediated AR signals promote bladder carcinogenesis by down-regulating the expression of UGTs in the bladder.
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Key words: androgen deprivation; chemical carcinogens; glucuronidation; knockout mouse model; SVHUC cell line

INTRODUCTION

Urinary bladder cancer is at least three times more common among males than females worldwide [1]. Excessive exposure to carcinogens, such as cigarette smoke and industrial chemicals, has been suggested to be a cause of higher incidence of bladder cancer in men. However, after controlling for these carcinogenic factors, men still have a substantially higher risk of bladder cancer than women [1,2]. We recently showed molecular evidence suggesting that androgen receptor (AR) signaling pathway promotes bladder carcinogenesis as well as cancer progression [3]. Castrated male and wild-type female mice had a lower incidence of bladder cancer induced by a chemical carcinogen, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), than wild-type male mice. In addition, AR knockout (ARKO) in male and female mice completely prevented bladder cancer development.

Industrial chemicals, such as aromatic amines, are well-known bladder carcinogens. They can be glucuronidated in the liver and excreted either by the biliary system into the intestine or via the blood into the urinary system [4]. In the bladder, their glucuronides can be hydrolyzed by the acidic urine or by bacterial β-glucuronidase, and the parent compounds may accumulate in the bladder epithelium. Then, these accumulated aromatic amines undergo further metabolism by peroxidation and/or *O*-acetylation to form DNA adducts that may initiate bladder carcinogenesis [5].

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most potent and abundant procarcinogens found in tobacco and tobacco smoke. NNK is rapidly metabolized to its carbonyl

reduction, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL can be detoxified by forming glucuronidated NNAL, which is readily excreted in the urine. Indeed, the level of urinary NNAL is used as a biomarker for environmental tobacco smoke [6] and has been reported to correlate with smoking status in bladder cancer patients [7].

UDP-glucuronosyltransferases (UGTs) belong to a superfamily of major phase II drug metabolism enzymes that catalyze the glucuronidation of numerous endobiotics and xenobiotics. All known human UGTs are divided into three subfamilies, UGT1A, UGT2A, and UGT2B, based on gene sequence homology [8]. There are 13 subtypes of human *UGT1A* gene, located on chromosome 2q37, consisting of 13 individual promoters and different first exons. Each exon 1 is combined to four common exons by alternative splicing, generating four pseudogenes and nine functional proteins (UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10). In contrast to the UGT1A, the UGT2 subfamily consists of individual gene cluster. In mouse, the genes are described in lower case after first letter. Mouse Ugt1a locus on chromosome 1C5/D contains 14 different first exons and a shared set of exons 2–5, generating five pseudogenes and nine functional genes (Ugt1a1, Ugt1a2, Ugt1a5, Ugt1a6a, Ugt1a6b, Ugt1a7c, Ugt1a8, Ugt1a9, and Ugt1a10) [9]. There is no clear consensus about the functional homology between human UGT and mouse Ugt.

The UGT1A superfamily, rather than UGT2A and UGT2B, generally plays a more important role in metabolism of aromatic amines. In the liver, each of UGT1A1, UGT1A4, or UGT1A9 is able to metabolize all of the aromatic amines tested, but UGT1A4 and UGT1A9 exhibit higher rates of metabolism [10]. UGT1A4 and UGT1A9 have also been shown to possess NNAL glucuronidation activity [11]. Although the liver is considered the most important organ for metabolism, including glucuronidation, UGTs are also expressed in some extrahepatic tissues, such as the gastrointestinal tract [12], kidney [13], and aerodigestive tract [14]. A recent study, using various human tissues, revealed that normal bladder expresses all the UGT subtypes except UGT2B17 [15]. Additionally, down-regulation of UGT1A expression was observed in several bladder cancer tissue samples, compared to normal urothelium [16,17].

Thus, UGT1As are likely key enzymes involved in detoxification of major bladder carcinogens. Interestingly, UGT1A has been identified as an androgen responsive gene in a prostate cancer cell line [18]. The purpose of this study was to investigate the relationship between AR signals and UGT1A/Ugt1a expression in the bladder. Alterations of UGT expression in bladder urothelium could be underlying mechanisms responsible for bladder carcinogenesis mediated via the AR signaling pathway.

MATERIALS AND METHODS

Cell Culture and Chemicals

Human urothelium cell line SVHUC and human embryonic kidney cell line 293T (both obtained from the American Type Culture Collection, Manassas, VA) were maintained in appropriate media (Mediatech, Manassas, VA; Kaighn's Modification of Ham's F-12 for SVHUC; Dulbecco's modified Eagle's medium for 293T) supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified atmosphere of 5% CO_2. Cells were cultured in phenol-red free medium supplemented with 5% charcoal-stripped FBS at least 18 h before experimental treatment. We obtained dihydrotestosterone (DHT) from Sigma (St. Louis, MO) and hydroxyflutamide (HF) from Schering (Kenilworth, NJ).

Stable Cell Line With AR

To establish a cell line stably expressing the AR, a lentivirus vector pWPI-AR/pWPI-control, psPAX2, and pMD2.G were first co-transfected into 293T cells using GeneJuice transfection reagent (Novagen, Gibbstown, NJ). Forty-eight hours after transfection, SVHUC cells were cultured in the presence of viral supernatant containing 8 μg/mL polybrene (Millipore, Billerica, MA) for 6 h. Flow cytometry was used to obtain pure SVHUC overexpressing AR (SVHUC-AR) or vector only (SVHUC-V).

Reporter Gene Assay

SVHUC cells at a density of 50–60% confluence in 24-well plates were co-transfected with 250 ng of MMTV-luc reporter plasmid DNA and 2.5 ng of pRL-TK-luc plasmid DNA, using GeneJuice. Six hours after transfection, the medium was replaced with medium supplemented with 5% charcoal-stripped FBS in the presence of ligands (DHT, HF, or both) for 24 h. Cells were harvested, lysed, and assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) and luminometer (TD-20/20, Turner BioSystems, Sunnyvale, CA).

Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction (PCR)

Total RNA (1.0 μg) isolated using TRIzol (Invitrogen, Carlsbad, CA) was reverse transcribed using 1 μmol/L oligo (dT) primers and four units of Omniscript reverse transcriptase (Qiagen, Valencia, CA) in a total volume of 20 μL. Real-time PCR was then performed in 15 μL system by using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously [3]. The primer sequences

are given in Table 1. Due to the high degree of homology, we were unable to design primers that allow to separately amplify Ugt1a6a and Ugt1a6b.

Western Blot

Protein extraction and western blot were performed, as described previously [19] with minor modifications. Briefly, equal amounts of protein (50 μg) obtained from cell extracts were separated in 10% sodium dodecylsulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore) by electroblotting using a standard protocol. Specific antibody binding was detected, using horseradish peroxidase detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, IL). An anti-AR (N20) antibody (diluted 1:2,000), an anti-UGT1A4 (L14) antibody (diluted 1:200), and an anti-β-actin antibody (diluted 1:1,000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

ARKO Mice

We created male ARKO mice in the background of the mosaic founder strain (CL57BL/6-129SV), as described previously [20]. Animal care was in accord with institutional guidelines. Male ARKO mice ($n = 6$) and their male wild-type littermates ($n = 3$) were allowed food and water ad libitum. Slow releasing pellets (1.5 mg DHT per mouse; Innovative Research of America, Sarasota, FL) were injected into half of the ARKO mice ($n = 3$) at 5 wk of age and replaced every 90 days. All mice were killed by administration of pentobarbital followed by rapid cervical dislocation at 40 wk of age,

and urinary bladder specimens were harvested. These specimens were rapidly frozen in liquid nitrogen and stored at $-80°C$ for subsequent RNA analysis.

Orchiectomized Mice

CL57BL/6-129SV mice (male: $n = 15$; female: $n = 6$) were housed according to the institutional guidelines and were allowed food and water ad libitum. Male mice received bilateral orchiectomy ($n = 9$) or sham surgery ($n = 6$) and female mice received sham surgery ($n = 6$) at 5 wk of age. Among castrated mice, 0.1 mL of peanut oil with ($n = 3$) or without ($n = 6$) 200 μg of DHT was injected subcutaneously every 2 days. One week after the surgery, all the mice were sacrificed and urinary bladders were harvested, as described above.

Bladder Tissue Microarray (TMA) and Immunohistochemistry

Appropriate approval from Institutional Review Board of the University of Rochester Medical Center was obtained prior to construction and use of the TMA. Bladder cancer TMA was constructed from formalin fixed paraffin embedded cystectomy specimens (24 tumors and 18 benign urothelial tissues from the same bladders with tumors), as described previously [21]. These 24 patients included 19 men and 5 women, with a mean age at cystectomy of 66.2 years (range: 49–86 years) and mean follow-up after surgery of 10.1 months (range 3–20 months). All the cases were histologically diagnosed as high-grade urothelial carcinoma. These included 2 pTis, 3 pT1, 4 pT2,

Table 1. Sequences of Primers Used for Real-Time RT-PCR Analysis

Primer	Sense	Anti-sense	Amplicon size
UGT1A1	5′-GCCATGCAGCCTGGAATT-3′	5′-GCCTGGGCACGTAGGA-3′	63
UGT1A3	5′-TGTTGAACAATATGTCTTTGGTCTA-3′	5′-CACATCAAAGGAAGTAGCATTCAG-3′	98
UGT1A4	5′-TTACGCTGGGCTACACTCA-3′	5′-GGCCAAAGATACATTGTTCAT-3′	95
UGT1A5	5′-CGCCTTTTGCTGGGTCACA-3′	5′-GATCAGGGCCTCATTATGCAGTAG-3′	144
UGT1A6	5′-CTGCCTCCTTCGCTCATT-3′	5′-CCAGTGGCTTCCGTCCT-3′	112
UGT1A7	5′-CGT AGT CAT GCC AGA GGT G-3′	5′-CGC AAT GGT GCC GTC C-3′	141
UGT1A8	5′-TGGATTTCGCCGATGCTCAAT-3′	5′-AAACTTCGTACTTGTGCTTTCCATTGAG-3′	43
UGT1A9	5′-TGAGGTCGGTGGTGGAG-3′	5′-TGAGCATGGGCAAAAGC-3′	169
UGT1A10	5′-TTACTCAACCTCGTACACTCTG-3′	5′-ACTTTGTGCCTGTGCTTTC-3′	85
Human GAPDH	5′-CTCCTCCACCTTTGACGCTG-3′	5′-CATACCAGGAAATGAGCTTGACAA-3′	77
Ugt1a1	5′-GCTGGGAGGCTGTTAGTGTT-3′	5′-GTGTATTGAGGCTTCAGGTGC-3′	123
Ugt1a2	5′-AACAGAACTTGGAGATGGACACG-3′	5′-GGAGCAGCAGCCCTGAGATT-3′	66
Ugt1a5	5′-TTGGACTGAAGGTGAGAAGGTGCT-3′	5′-TGGTGACCTTGGGCGTGGA-3′	99
Ugt1a6a/b	5′-GGCTGATGGTGGCTGACTGG-3′	5′-GGCCCTTGAGCAAGAGTAAGAAAT-3′	58
Ugt1a7c	5′-ATGGGAGCCACTGGTTTACGAT-3′	5′-TTCGTCAGCTGCCAACTCACC-3′	103
Ugt1a8	5′-GCTCACAAGCAATGGGAAAATCG-3′	5′-ACTTGGAAGAAACCTTTGCCTGAAT-3′	77
Ugt1a9	5′-GGATTACTCTGGGCTCTGATTTTA-3′	5′-AAGGAGGTGGGAAAGGCTACA-3′	55
Ugt1a10	5′-TCCCCAGACCTGTGATGCC-3′	5′-CTTCCCCTGGAGACAGTTCATC-3′	107
Mouse β-actin	5′-AGTGTGACGTTGACATCCGTA-3′	5′-GCCAGAGCAGTAATCTCCTTC-3′	112

and 15 ≥pT3 tumors, as well as 12 node-negative and 12 node-positive tumors. Immunohistochemical staining was then performed, as described previously [19,21] with minor modifications. Briefly, TMA sections (4 μm thick) were deparaffinized in xylene, rehydrated in a graded ethanol series, and incubated in 3% hydrogen peroxide to block endogenous peroxidase. Slides were incubated overnight at 4°C with anti-AR (N20) antibody (diluted 1:100) and anti-UGT1A (H300) antibody (diluted 1:100; Santa Cruz Biotechnology). We then incubated the samples with a broad spectrum secondary antibody (Invitrogen). After being rinsed by phosphate-buffered saline, the slides were incubated with diaminobenzidine (Invitrogen), and finally counterstained with hematoxylin. These stains were manually scored by one pathologist (H.M.) blinded to patient identity. German Immunoreactive Score (0–12) was calculated, separately in benign and malignant tissues, by multiplying the percentage of immunoreactive cells (0% = 0; 1–10% = 1; 11–50% = 2; 51–80% = 3; 81–100% = 4) by staining intensity (negative = 0; weak = 1; moderate = 2; strong = 3). Scores were considered negative (−; 0–1), weakly positive (1+; 2–4), moderately positive (2+; 6–8), and strongly positive (3+; 9–12).

Statistical Analysis

Student's *t*-test was used to analyze differences in mRNA expression levels of UGT/Ugt between the two groups. Fisher's exact test and the chi-square test were used to analyze differences in UGT1A expression in bladder TMA. Survival rates in patients were calculated by the Kaplan–Meier method and comparison was made by log-rank test. P values less than 0.05 were considered to be statistically significant.

<center>RESULTS</center>

Expression and Transcriptional Activity of AR in SVHUC Cells

Because our preliminary study indicated human normal urothelium cell line SVHUC lacked AR, a human full-length wild-type AR was stably expressed in the cells by lentivirus. Then, we tested AR expression in SVHUC-AR and SVHUC-V with treatment of DHT and/or antiandrogen HF. As shown in Figure 1A, strong expression of AR protein was confirmed in SVHUC-AR, but not in SVHUC-V. AR expression in SVHUC-AR was enhanced by DHT treatment and antagonized by HF, which by itself showed marginal effects. Luciferase activity was also determined in these cell extracts with transfection of a plasmid containing an androgen response element as a reporter of AR-mediated transcriptional activity. As shown in Figure 1B, DHT treatment increased luciferase
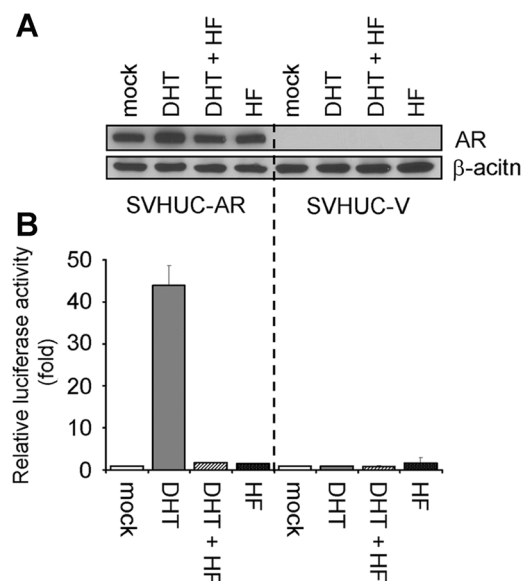

Figure 1. AR expression and transcriptional activity in SVHUC-AR and SVHUC-V cells. (A) SVHUC-AR and SVHUC-V were treated with ethanol (mock), 1 nM DHT, and/or 1 μM HF for 48 h, as indicated. Equal amounts of protein extracted from each cell line were immunoblotted for AR (110 kDa). β-Actin (43 kDa) served as the internal control. (B) SVHUC-AR and SVHUC-V were transfected with MMTV-Luc and cultured for 24 h in the presence of ethanol (mock), 1 nM DHT, and/or 1 μM HF, as indicated. Luciferase activity analyzed in a luminometer is presented relative to that of mock treatment in each cell line (first lanes; set as onefold). Each value represents the mean + SD from at least three independent experiments.

activity by 44-fold over mock treatment in SVHUC-AR, and HF showing only marginal activity clearly blocked the DHT effect. In SVHUC-V, DHT and HF showed only marginal effects on AR transcriptional activity. These data indicate that SVHUC-AR, but not SVHUC-V, possesses an active AR.

Androgen/AR-Mediated Down-Regulation of UGT1A in SVHUC Cells

We first tested mRNA expression of all the UGT1A subtypes in SVHUC-AR in the presence or absence of DHT by a quantitative real-time RT-PCR method. As shown in Figure 2A, DHT treatment showed 19–75% decrease in the levels of UGT1As in SVHUC-AR, compared to mock treatment.

Next, we further studied the expression of UGT1A1, UGT1A4, and UGT1A9, because these subtypes have been proved to be important to detoxify bladder carcinogens, including aromatic amines and NNAL [10,11]. SVHUC-AR and SVHUC-V were treated with DHT and/or HF and mRNA expression of these three UGT1A subtypes were determined. As shown in Figure 2B–D, DHT treatment reduced the levels of UGT1A1, UGT1A4, and UGT1A9 by 31%, 31%, and 63%, respectively,
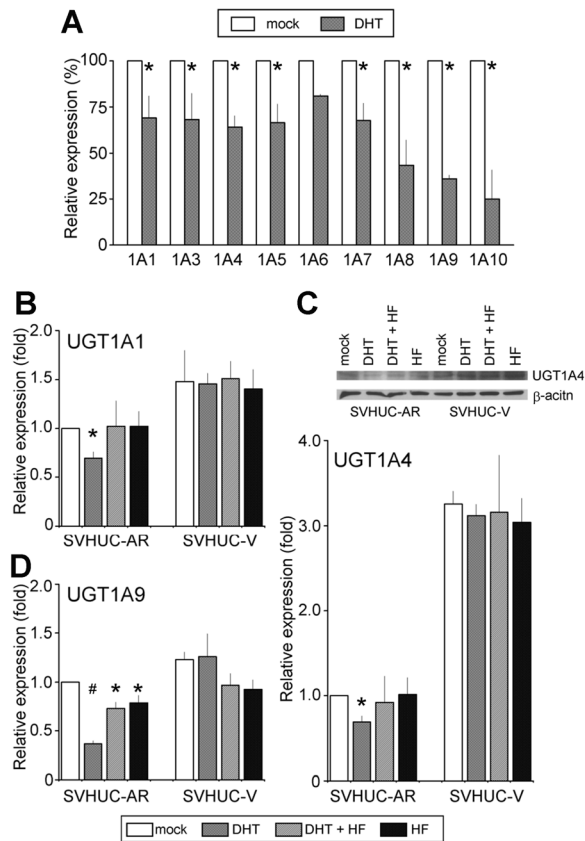
Figure 2. Regulation of UGT1A expression by androgen and AR in SVHUC cells. (A) SVHUC-AR cultured in the presence of ethanol (mock) or 1 nM DHT for 48 h was analyzed on real-time RT-PCR for all the subtypes of UGT1A. SVHUC-AR and SVHUC-V cultured in the presence of ethanol (mock), 1 nM DHT, and/or 1 μM HF for 48 h were analyzed on real-time RT-PCR for UGT1A1 (B), UGT1A4 (C, lower), and UGT1A9 (D). Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that of mock treatment in SVHUC-AR [first lanes; set as 100% (A) or onefold (B–D)]. Each value represents the mean + SD from at least three independent experiments. (C, upper) UGT1A4 protein expression. Equal amounts of protein extracted from each cell line were immunoblotted for UGT1A4 (56 kDa). β-Actin (43 kDa) served as the internal control. *$P < 0.05$ (vs. mock treatment in the same cell line). #$P < 0.01$ (vs. mock treatment in the same cell line).

over mock treatment in SVHUC-AR. By contrast, DHT showed only marginal effects ($<5\%$ changes) in SVHUC-V. On UGT1A1 and UGT1A4, HF showed only marginal effects in SVHUC-AR and SVHUC-V and it definitely blocked DHT effect in SVHUC-AR. On UGT1A9, HF itself showed 21% and 25% reduction in SVHUC-AR and SVHUC-V, respectively, and it partially antagonized DHT effect in SVHUC-AR. When compared with SVHUC-AR, SVHUC-V showed higher levels of these three UGT1A subtypes in any treatment group. In the presence of DHT, levels of UGT1A1, UGT1A4, and UGT1A9 in SVHUC-V were higher (2.1-, 4.6-, and 3.4-fold, respectively) than those in SVHUC-AR.

We also tested protein expression of UGT1A4 in SVHUC-AR and SVHUC-V treated with DHT and/

or HF. As expected, similar changes in UGT levels were observed (Figure 2C, upper). These findings suggest that AR signals down-regulate UGT1As, even without androgens, in human normal urothelial cells.

Expression of Ugt1a in ARKO Mouse Bladder

To further investigate the involvement of AR signals in the regulation of Ugt1a expression in vivo, Ugt1a levels were analyzed in the bladders from ARKO mice. As shown in Figure 3, mRNA expression of all the Ugt1a subtypes was up-regulated in ARKO mice (1.4- to 3.5-fold) over wild-type male littermates. Although ARKO mice are known to have low levels of androgens [3,20], DHT supplement in these animals did not alter Ugt1a expression levels. These results further suggest that AR signals repress Ugt1a expression in mouse bladder and the effects of androgens on Ugt1a are mediated through the AR.

Expression of Ugt1a in Castrated Mouse Bladder

We then tested androgen effects on Ugt1a expression in male mouse bladder and also compared Ugt1a expression between male and female mice. Wild-type mice underwent bilateral orchiectomy or sham surgery followed by androgen or mock treatment. As shown in Figure 4, some Ugt1a subtypes, especially Ugt1a8, Ugt1a9, and Ugt1a10 (2.3-, 2.0-, and 1.6-fold, respectively), were up-regulated by castration in male mice. DHT supplement clearly eliminated the effect of castration on these three subtypes but induced marginal changes ($<13\%$) in the levels of other subtypes. Of note were higher expression levels of all the subtypes of
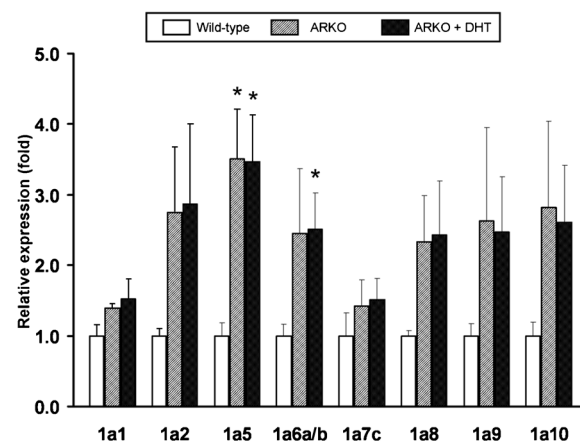

Figure 3. Effects of ARKO on Ugt1a expression in mice. At 40 wk of age, urinary bladder from male wild-type mice ($n = 3$), ARKO mice ($n = 3$), and ARKO mice with DHT treatment ($n = 3$) were harvested and analyzed on real-time RT-PCR for all the subtypes of Ugt1a. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that in the wild-type (first lanes; set as onefold). Each value represents the mean + SE. *$P < 0.05$ (vs. wild-type male).
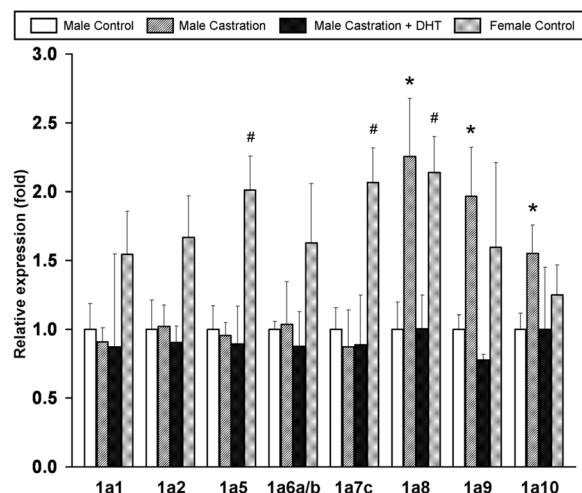
Figure 4. Effects of castration on Ugt1a expression in mice. Wild-type male mice received sham surgery ($n = 6$), surgical castration ($n = 6$), or surgical castration followed by DHT supplement ($n = 3$) at 5 wk of age. Wild-type female mice received sham surgery ($n = 6$) at 5 wk of age. One week after the surgery, urinary bladders were harvested and analyzed on real-time RT-PCR for all the subtypes of Ugt1a. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that in wild-type males with sham surgery (first lanes; set as onefold). Each value represents the mean + SE. *$P < 0.05$ (vs. male control). #$P < 0.01$ (vs. male control).

Ugt1a in female mice (1.3- to 2.1-fold over wild-type males).

Immunoreactivity of UGT1A in Normal Bladder and Bladder Cancer Tissue Samples

We performed immunohistochemical stains for UGT1A in 24 radical cystectomy specimens with high-grade urothelial carcinoma. Positive signals were detected predominantly in cytoplasm of epithelial cells (Figure 5A–D). The results of UGT1A expression in tissue samples are summarized in Table 2. Overall, all the non-neoplastic and neoplastic bladders showed at least weak signals in urothelial cells. Strong signals were found in 11 (46%) of 24 cancer tissues and in 17 (94%) of 18 corresponding benign tissues. Thus, the expression of UGT1A was significantly weaker in urothelial carcinoma than in benign urothelium ($P = 0.0009$). There were no statistically significant correlations between the intensity of UGT1A expression and gender, presence of muscle invasion ($\leq$T1 vs. $\geq$T2), or lymph node metastasis. In the 24 bladder cancer specimens where AR expression had also been immunohistochemically analyzed [21], 9 of 16 (56%) AR-negative tumors showed strong UGT1A expression and 6 of 8 (75%) AR-positive tumors showed weak/moderate UGT1A expression. Thus, there was a tendency of inverse correlation between expressions of AR and UGT1A, but it was not statistically significant ($P = 0.1557$).
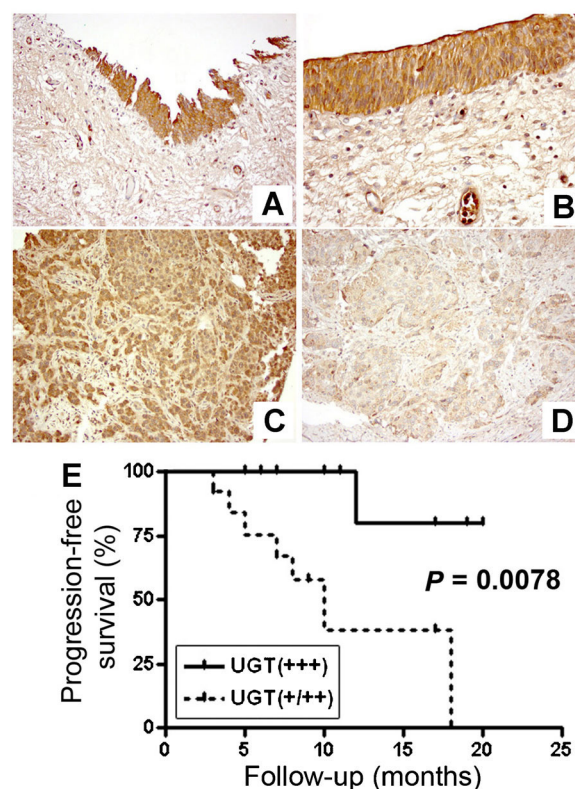

Figure 5. Immunohistochemistry of UGT1A in benign and malignant bladder tissues. All benign bladder epithelia (A and B), except one case, showed a strong UGT1A immunoreactivity. In bladder carcinomas, strong (C) to weak (D) stains for UGT1A were observed. The wall of vessels was also positive for UGT1A, serving as a positive control. Original magnification, 200× (A, C, and D) or 400× (B). (E) Kaplan–Meier analysis of progression-free survival according to strong UGT1A expression. Comparison was made by log-rank test.

To assess possible associations between UGT1A staining and disease progression, we performed Kaplan–Meier analysis coupled with log-rank test. Of 24 patients with a mean follow-up of 10.1 months, 8 (33%) developed recurrent/metastatic tumors after radical surgery. As shown in Figure 5E, weak or moderate positivity of UGT1A was significantly associated with tumor progression ($P = 0.0078$).

DISCUSSION

UGTs play a major role in the elimination of numerous carcinogens by: (i) transportation of the ultimate carcinogens excreted via the biliary or urinary tract; and (ii) sequestration of proximate carcinogens, leading to their detoxification [22]. The urinary bladder involves the main pathway for excretion of glucuronides and expresses all the subtypes of UGT except UGT2B17 [15]. Accumulating evidence has indicated that various transcription factors, such as hepatic nuclear factor-1α (HNF1α), HNF4α, arylhydrocarbon receptor (AhR),

Table 2. Expression of UGT1A in Tumor and Benign Bladder Tissue Microarrays

	Positive score		
	1–2+ (%)	3+ (%)	Fisher's exact test
Benign (n = 18)	1 (6)	17 (94)	P = 0.0009
Cancer (n = 24)	13 (54)	11 (46)	
Sex			P = 0.2157
Male (n = 19)	9 (47)	10 (53)	
Female (n = 5)	4 (80)	1 (20)	
Stage (pT)			P = 0.4146
≤T1 (n = 5)	2 (40)	3 (60)	
≥T2 (n = 19)	11 (58)	8 (42)	
Lymph node metastases			P = 0.2068
Negative (n = 12)	5 (42)	7 (58)	
Positive (n = 12)	8 (67)	4 (33)	
AR expression			P = 0.1557
Negative (n = 16)	7 (44)	9 (56)	
Positive (n = 8)	6 (75)	2 (25)	

and erythroid 2-related factor (Nrf2), have an impact on the activity of *UGT* genes [22]. In mouse urinary bladder, it was shown that BBN treatment reduced Ugt1a expression in a time and dose dependent manner and AhR signaling pathway was associated with this down-regulation [23,24]. It was also reported that knock-out of Nrf2 reduced Ugt1a expression and increased cancer incidence in BBN treated mice [23]. These data suggest that UGT in the urinary bladder functions to protect against chemical carcinogens. In the current study, we focused on UGT1A, especially UGT1A1, UGT1A4, and UGT1A9, that are known to be important in detoxifying bladder carcinogens, such as aromatic amines [10] and NNAL [11] in the liver. Nonetheless, because there is no clear functional correspondence between human UGT and mouse Ugt, the results on mouse model should be interpreted with caution.

Ligand activated nuclear receptors have been shown to regulate UGT expression [25]. Among them, AR was responsible for the gender difference in expression of some Ugt subtypes in mice [26] and rats [27]. Androgen itself is metabolized by some UGT subtypes and negative regulations of these enzymes by androgen have been reported [28]. In addition, a genome wide search identified UGT1A as a novel AR regulated gene in the prostate [18]. AR is also shown to suppress AhR activity by forming a complex [29]. Because AR, as a ligand-regulated transcription factor, likely promotes the development and progression of bladder cancer [3,30,31], these experimental observations formed the basis of our hypothesis: androgens regulate UGT1A expression in the bladder via the AR pathway, leading to male dominance in bladder cancer incidence. Although an AR-binding site (ARBS) was found in the non-promoter regions of

UGT1As, it could influence the expression of only UGT1A1 and UGT1A3 whose transcriptional start sites are relatively close (>17 kb) to the ARBS [18]. We were unable to identify any putative ARBSs in each promoter region of UGT1A by a computer analysis, suggesting that ARBS(s) distant from the promoter region contribute to UGT1A regulation.

We first showed all the subtypes of UGT1A were down-regulated by androgen in the normal urothelial cell line SVHUC overexpressing the AR. The magnitudes of down-regulation are larger in UGT1A8, UGT1A9, and UGT1A10, compared to the others. It was likely that androgen functions through the AR pathway because an antiandrogen HF, at least partially, antagonized the effect of androgen in SVHUC-AR and no androgen effect was observed in SVHUC-V lacking a functional AR. In addition, HF down-regulated UGT1A9 in both SVHUV-AR and SVHUC-V cells. It has been reported that flutamide is glucuronidated by UGT [32] and is one of AhR activators [33]. Therefore, flutamide itself may regulate UGT1A9 via the pathway(s) other than AR. Of note were higher basal levels of UGT1As, particularly UGT1A4, in SVHUC-V than in SVHUC-AR, suggesting down-regulation of UGT1As via non-androgen-mediated AR signals.

We then analyzed Ugt1a expression by using ARKO and castrated mouse models. As expected, all the Ugt1a subtypes were up-regulated in the bladders from ARKO mice with or without androgen supplement. However, castration resulted in increases in the expression of only Ugt1a8, Ugt1a9, and Ugt1a10, and androgen supplement eliminated this effect. These results, together with the data in cell lines, suggest that androgen is necessary for AR-mediated down-regulation of some UGT1A/Ugt1a subtypes, but AR signals induced by sub-physiological levels of androgens sufficiently

down-regulate the other subtypes. Interestingly, female mice had higher expression of all the subtypes of Ugt1a. Since AR expression status in human bladder is similar between both sexes [34–36], ligand effects as well as those other than AR signals may contribute to gender difference in UGT expression. It has been reported that estradiol up-regulates some UGT subtypes in liver [37] and breast [38] cancer cell lines. Underlying mechanisms responsible for this gender difference need to be further explored.

In bladder cancer, the levels of UGT1A transcript were lower than those in normal counterpart [16]. An immunohistochemical study further revealed that UGT1A was strongly expressed in cytoplasm of normal bladder urothelium, whereas UGT1A expression was significantly decreased in urothelial carcinoma and was virtually negative in some high-grade tumors [17]. We confirmed UGT1A down-regulation in high-grade bladder cancer tissues, compared to non-neoplastic urothelium by immunohistochemistry. The present study also analyzed and compared the prognostic value of UGT expression by using Kaplan–Meier survival curves and log-rank test and expression status of UGT1A was found to significantly correlate with tumor progression. Interestingly, loss of strong UGT1A expression was observed in 47% of male patients versus 80% of female patients, although the difference was not statistically significant. The data may indeed support clinical evidence indicating women tend to present with less favorable tumor characteristics than men [39]. Overall, our immunostaining results suggest that UGT1A loss in bladder cancer could predict worse outcome.

We recently assessed AR expression, using the same bladder TMA, and found a strong correlation between AR positivity and tumor progression [21]. Expressions of AR and UGT, although there was no statistically significant difference, tended to be inversely correlated. These data may suggest that UGT1A down-regulated by AR signals also plays a preventive role in cancer progression. However, the sample size in our immunohistochemical study was relatively small, and, therefore, additional work with larger patient cohorts, including different grades of bladder tumor, should be done to conclude the relationship between AR and UGT expressions.

In conclusion, we showed down-regulation of UGT1A by androgen/AR in human normal bladder urothelial cells as well as up-regulation of Ugt1a in ARKO and castrated mouse bladders. Bladder cancer also exhibited lower levels of UGT1A expression, compared to normal urothelium. These results suggest that androgen-mediated AR signals play an important role in bladder cancer initiation by down-regulating the expression of UGT1As. Further functional analyses of UGT in normal bladder/bladder cancer are necessary to determine their biological significance.

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Androgen activates β-catenin signaling in bladder cancer cells

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Abstract

Androgen receptor (AR) signals have been implicated in bladder carcinogenesis and tumor progression. Activation of Wnt/β-catenin signaling has also been reported to correlate with bladder cancer progression and poor patients' outcomes. However, cross talk between AR and β-catenin pathways in bladder cancer remains uncharacterized. In radical cystectomy specimens, we immunohistochemically confirmed aberrant expression of β-catenin especially in aggressive tumors. There was a strong association between nuclear expressions of AR and β-catenin in bladder tumors ($P=0.0215$). Kaplan–Meier and log-rank tests further revealed that reduced membranous β-catenin expression ($P=0.0276$), nuclear β-catenin expression ($P=0.0802$), and co-expression of nuclear AR and β-catenin ($P=0.0043$) correlated with tumor progression after cystectomy. We then assessed the effects of androgen on β-catenin in AR-positive and AR-negative bladder cancer cell lines. A synthetic androgen R1881 increased the expression of an active form of β-catenin and its downstream target c-myc only in AR-positive lines. R1881 also enhanced the activity of β-catenin-mediated transcription, which was abolished by an AR antagonist hydroxyflutamide. Using western blotting and immunofluorescence, R1881 was found to induce nuclear translocation of β-catenin when co-localized with AR. Finally, co-immunoprecipitation revealed androgen-induced associations of AR with β-catenin or T-cell factor (TCF) in bladder cancer cells. Thus, it was likely that androgen was able to activate β-catenin signaling through the AR pathway in bladder cancer cells. Our results also suggest that activation of β-catenin signaling possibly via formation of AR/β-catenin/TCF complex contributes to the progression of bladder cancer, which may enhance the feasibility of androgen deprivation as a potential therapeutic approach.

Introduction

The androgen receptor (AR), a member of the nuclear receptor superfamily, mediates most of its physiological functions through transcriptional activation of downstream genes by binding to androgens (Heinlein & Chang 2004). In the presence of androgens, the AR located in the cytoplasm dissociates from heat-shock protein and translocates to the nucleus, leading to regulation of the target genes. AR and other nuclear receptors have been detected in the urothelium and/or stromal cells of the urinary bladder, and emerging data suggest that bladder cancer is

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an endocrine-related neoplasm (reviewed in Li et al. (2012) and Miyamoto et al. (2012)). AR signals have been implicated in bladder carcinogenesis and tumor progression. Specially, promising evidence further documents a critical role of AR in bladder cancer cell proliferation (Miyamoto et al. 2007, Johnson et al. 2008, Boorjian et al. 2009, Wu et al. 2010, Zheng et al. 2011). Nonetheless, the mechanism by which AR signaling modulates bladder cancer progression remains poorly understood.

The canonical Wnt/β-catenin signaling pathway has been shown to play a pivotal role in normal cell growth and differentiation, embryonic development, and apoptosis (Morin 1999, Polakis 1999, Vlad et al. 2008). It is also proposed to be involved in the development of urogenital system (Lako et al. 1998). As a key component of the Wnt signaling pathway, β-catenin is a multifunctional protein and has two major pools: a membrane pool, required for cell–cell adhesion, and cytoplasmic/nuclear pool, responsible for Wnt/β-catenin signal transduction (Miller & Moon 1996). In the absence of a Wnt signal, β-catenin is usually maintained at a low level because of being constitutively degraded via the ubiquitin proteasome pathway. Wnt signaling inhibits this process, leading to cytosolic β-catenin accumulation. Subsequently, it translocates to the nucleus, forms complexes with members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors, and thereby activates target genes, such as the proto-oncogene c-MYC, the cell cycle activator cyclin-D1, and the epidermal growth factor receptor (EGFR; Behrens et al. 1996, Brabletz et al. 2000, Tan et al. 2005). Thus, growing evidence suggests an important role of Wnt/β-catenin signaling in cell proliferation and differentiation in various types of human malignancies (Miller & Moon 1996, Polakis 2000, Lustig & Behrens 2003, Gavert & Ben-Ze'ev 2007).

Using bladder tissue specimens, differential expression of some genes encoding Wnt proteins has been detected in normal bladders, superficial tumors, and invasive tumors (Bui et al. 1998). Both downregulation of the Wnt antagonists (Hsieh et al. 2004, Stoehr et al. 2004) and upregulation of the Wnt target genes (Shiina et al. 2002) have been observed in bladder cancer tissues. It has also been shown that nuclear accumulation of β-catenin correlates with worse outcomes in patients with bladder cancer (Kastritis et al. 2009). These observations suggest that Wnt signaling is active in advanced urothelial tumors. Of note, the application of various small molecules that target the Wnt/β-catenin signaling pathway led to inhibition of bladder cancer cell proliferation (Urakami et al. 2006, Tang et al. 2009, Hirata et al. 2012).

It has been well documented in several cancers that Wnt/β-catenin and AR signaling pathways are closely related. It has shown, for instance, that the AR can be activated through the Wnt/β-catenin pathway in castration-resistant prostate cancer (Wang et al. 2008). However, the possible convergence between these two pathways in bladder cancer remains largely unknown. In this study, we focus on investigating the effects of androgens on β-catenin signals in AR-positive and AR-negative bladder cancer cells. To the best of our knowledge, this is the first report to show androgens/AR-mediated activation of Wnt/β-catenin signaling in bladder cancer cells.

Materials and methods

Bladder tissue microarray and immunohistochemistry

Bladder tissue microarray (TMA) was constructed from 24 formalin-fixed paraffin-embedded cystectomy specimens retrieved from the Surgical Pathology archives, as described previously (Zheng et al. 2011, Izumi et al. 2013). Appropriate approval from the Institutional Review Board of the University of Rochester Medical Center was obtained before construction and use of the TMA. These 24 patients included 19 men and five women, with a mean age at cystectomy of 66.2 years (range: 49–86 years) and a mean follow-up after the surgery of 11.4 months (range: 3–24 months). All the tumors were histologically diagnosed as high-grade urothelial carcinoma. These included 5 ≤pT1, 19 ≥pT2, 12 pN0, and 12 pN+ tumors.

Immunohistochemical staining was performed on the sections (5 μm thick) from the bladder TMA, as described previously (Zheng et al. 2011, Izumi et al. 2013), with minor modifications. Briefly, tissues were deparaffinized in xylene, rehydrated in a graded ethanol series, and incubated in 3% hydrogen peroxide to block endogenous peroxidase. Slides were incubated overnight at 4 °C with an anti-AR (clone N20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or an anti-β-catenin antibody (clone β-catenin-1, Dako, Carpinteria, CA, USA). The samples were then incubated with a broad-spectrum secondary antibody (Invitrogen, Carlsbad, CA, USA). After being rinsed in PBS, the slides were incubated with diamino-benzidine (Invitrogen) and finally counterstained with hematoxylin. These stains were manually quantified by one pathologist (H M) blinded to sample identity. The expression of β-catenin in cancer cells was classified as Hu et al. (2011) described: >70% of cell membranes

stained as normal, otherwise as reduced, and >10% of nuclei or cytoplasms stained as positive.

Cell culture and chemicals

Human urothelial carcinoma cell lines, UMUC3, 5637, and J82, obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in DMEM (Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. At least 18 h before experimental treatment, cells were cultured in phenol red-free DMEM (Mediatech) supplemented with 5% charcoal-stripped FBS. Methyltrienolone (R1881) was purchased from PerkinElmer (Waltham, MA, USA), and hydroxy-flutamide (HF) was from Schering (Kenilworth, NJ, USA).

Stable cell lines with AR and AR-short hairpin RNA

Cell lines stably expressing a full-length wild-type human AR (5637-AR and J82-AR) or vector only (5637-V and J82-V) were established using a lentivirus vector (pWPI-AR or pWPI-control) with psPAX2 envelope and pMD2.G packaging plasmids, as we described previously (Zheng et al. 2011, Izumi et al. 2012). Similarly, stable AR knock-down/control cell lines (UMUC3-AR-short hairpin RNA (shRNA)/UMUC3-control-shRNA) were established with a retrovirus vector pMSCV/U6-AR-shRNA or pMSCV/U6-control-shRNA (Miyamoto et al. 2007, Zheng et al. 2011).

Reporter gene assay

Cells at a density of 50–60% confluence in 24-well plates were co-transfected with 250 ng Topflash reporter plasmid DNA (plasmid 12456 M50 Super 8×TOPFlash containing 7 TCF/LEF binding sites, Addgene, Cambridge, MA, USA) or a control Fopflash reporter plasmid DNA (plasmid 12457 M51 Super 8×FOPFlash containing six mutated TCF/LEF binding sites, Addgene) along with 2.5 ng pRL-TK renilla luciferase plasmid DNA, using GeneJuice transfection reagent (Novagen, Gibbstown, NJ, USA). After 6 h of transfection, the cells were cultured in the presence or absence of ligands (R1881, HF, or both) for 24 h. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) and luminometer (TD-20/20, Turner BioSystems, Sunnyvale, CA, USA). The ratio of Topflash:Fopflash values normalized to renilla was used as an indicator of Wnt/β-catenin activity.

Western blot

Protein extraction and western blot were performed, as described previously (Izumi et al. 2012), with minor modifications. Separate cytoplasmic and nuclear protein fractions were obtained, using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein obtained from cell extracts were separated by 10–12% SDS–PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) by electroblotting using a standard protocol. Specific antibody binding was detected using an anti-AR (clone N20; diluted 1:2000), an anti-β-catenin antibody (clone 14/β-catenin; diluted 1:2000; BD Bioscience, Franklin Lakes, NJ, USA), an anti-active-β-catenin (clone 8E7; diluted 1:1000; Millipore), an anti-c-myc antibody (clone Y69; diluted 1:1000; Epitomics, Burlingame, CA, USA), an anti-Histone H1 antibody (clone FL-219; diluted 1:1000; Santa Cruz Biotechnology), or an anti-GAPDH antibody (clone 6C5; diluted 1:1000; Santa Cruz Biotechnology), with HRP detection system (Super-Signal West Pico Chemiluminescent Substrate; Thermo Scientific).

RT and real-time PCR

Total RNA (1.0 μg) isolated from cultured cells, using TRIzol (Invitrogen), was reverse transcribed using 1 μmol/l oligo (dT) primers and four units of Omniscript reverse transcriptase (Qiagen) in a total volume of 20 μl. Real-time PCR was then performed in 15 μl system using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously (Zheng et al. 2011). The primer sequences are given as follows: β-catenin (forward, 5′-AAGTGGGGTGGTA-TAGAGGCTCTTG-3′; reverse, 5′-GATGGCAGGCTCAGT-GATGTC-3′) and c-myc (forward, 5′-ACCAGATCCCGGA-GTTGGAA-3′; reverse, 5′-CGTCGTTTCCGCAACAAGTC-3′). GAPDH (forward, 5′-AAGGTGAAGGTCGGAGTCAAC-3′; reverse, 5′-GGGGTCATTGATGGCAACAATA-3′) was used as an internal control.

Co-immunoprecipitation

Cells were treated with ethanol or R1881 for 24 h, and protein (500 μg) from the cell lysates was incubated with 2 μg anti-AR rabbit polyclonal antibody (clone N20)/anti-β-catenin mouse MAB (clone 14/β-catenin) or normal rabbit/mouse IgG (Santa Cruz Biotechnology) overnight at 4 °C with agitation. To each sample, we added 20 μl protein A/G-agarose beads (Santa Cruz Biotechnology), incubated for 2 h, and washed four to five times with

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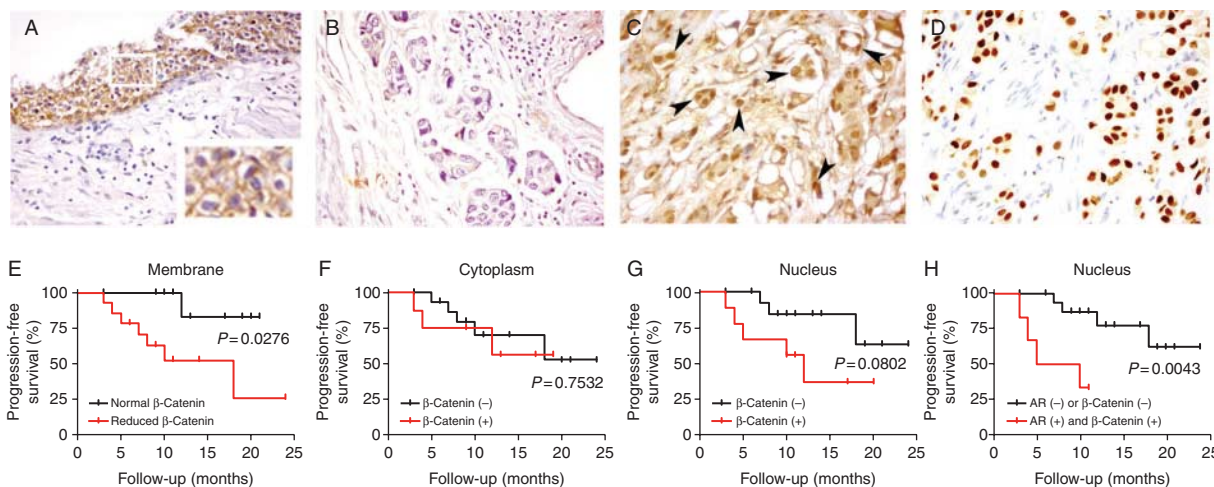
Figure 1

IHC of β-catenin (A, B and C) and AR (D) in bladder tissues (magnification: ×400) and progression-free survival rates (E, F, G and H) in patients with high-grade urothelial carcinoma. (A) β-Catenin expression in cell membrane of benign urothelium. (B) Reduced membranous β-catenin expression in urothelial carcinoma. Predominant nuclear expression of β-catenin (C; arrowheads) and AR (D) in the same tumor. Kaplan–Meier analysis was performed according to the expression of membranous β-catenin (E), cytoplasmic β-catenin (F), nuclear β-catenin (G), or nuclear β-catenin and AR (H), and comparisons were made by log-rank test.

radioimmunoprecipitation assay buffer. Then, we resolved the complex on a 10–12% SDS–PAGE, transferred to the membrane, and blotted with an anti-AR antibody (clone N20; diluted 1:2000), an anti-β-catenin antibody (clone 14/β-catenin; diluted 1:2000), or an anti-TCF4 antibody (clone EP2033Y; diluted 1:2000; Millipore).

Immunofluorescent staining

Cells were plated onto chamber slides (eight-well Thermo Scientific Nunc Lab-Tek) for immunostaining. After 12 h of seeding, the cells were cultured in DMEM with 5% charcoal-stripped FBS containing ethanol or R1881 for 24 h. At the end of drug treatment, culture medium was aspirated from each well, and the adherent cells were rinsed thrice with PBS and then fixed by 4% paraformaldehyde for 15 min at room temperature. After being washed with 0.1 M glycine for 20 min, the slides were kept in 1% Triton X-100 for 20 min at room temperature. Then, the cells were blocked with blocking buffer for 1 h at 37 °C. A primary antibody was incubated at 4 °C overnight, and Alexa 488- or 568-conjugated secondary antibody (diluted 1:200, Invitrogen) was added for 1 h at 37 °C. DAPI was used to visualize nuclei. Fluorescence images were acquired with an Olympus FV1000 confocal microscope. The number of nuclear staining per visual field was quantified in five randomly selected visual fields per chamber (total 900 cells) by a single observer who was unaware of the treatment group for the cells.

Statistical analyses

Student's t-test was used to analyze differences in relative Top/Fop luciferase activities and relative numbers of immunofluorescent staining between the two groups. Differences in protein expression between the two groups from human tissue samples were analyzed by Fisher's exact test. All these statistical tests were two sided. Progression-free survival rates in patients were calculated by the Kaplan–Meier method and comparison was made by log-rank test. P value < 0.05 was considered statistically significant.

Results

Immunoreactivity in bladder cancer tissue samples

We performed immunohistochemical stains for β-catenin in 24 radical cystectomy specimens of high-grade urothelial carcinoma. Coexisting benign urothelium exhibited β-catenin reactivity mainly with a membranous staining pattern (Fig. 1A), whereas positive signals were also detected in the nucleus and/or cytoplasm of some cancer cells. The expression of membranous and nuclear β-catenin in relation to pathological stages is summarized in Table 1. Overall, 15 (67.5%), eight (33.3%), and nine (37.5%) cancer cases showed reduced membranous expression (Fig. 1B), ectopic cytoplasmic expression, and ectopic nuclear expression (Fig. 1C) respectively. Deep

Table 1 Expression of β-catenin in bladder tissue microarrays.

	n	Membrane			Cytoplasm			Nucleus		
		Normal	Reduced[a]	P value[b]	Negative	Positive[c]	P value[b]	Negative	Positive[c]	P value[b]
All cases	24	9 (37.5%)	15 (62.5%)		16 (66%)	8 (33%)		15 (62.5%)	9 (37.5%)	
Stage (pT)				0.0474			1.0000			0.3256
≤pT1	5	4 (80%)	1 (20%)		3 (60%)	2 (40%)		2 (40%)	3 (60%)	
≥pT2	19	5 (26%)	14 (74%)		13 (68%)	6 (32%)		13 (68%)	6 (32%)	
Lymph node metastasis				0.4003			0.6668			1.0000
pN0	12	6 (50%)	6 (50%)		9 (75%)	3 (25%)		8 (67%)	4 (33%)	
pN+	12	3 (25%)	9 (75%)		7 (58%)	5 (42%)		7 (58%)	5 (42%)	
AR expression				0.1782			1.0000			0.0215
Negative	16	8 (50%)	8 (50%)		11 (69%)	5 (31%)		13 (81%)	3 (19%)	
Positive	8	1 (13%)	7 (87%)		5 (62.5%)	3 (37.5%)		2 (25%)	6 (75%)	

[a]<30% of cancer cells showed immunoreactivity.
[b]Fisher's exact test.
[c]>10% of cancer cells showed immunoreactivity.

invasion (≥pT2) was strongly associated with reduced membranous β-catenin compared with non-muscle-invasive tumors ($P=0.0474$), but not with cytoplasmic ($P=1.0000$) or nuclear ($P=0.3256$) β-catenin. There were no statistically significant differences in the expression pattern of β-catenin between male vs female tumors and between node-negative vs node-positive tumors. AR expression had also been immunohistochemically analyzed in these 24 bladder cancer specimens, including eight (33.3%) AR-positive cases (Fig. 1D; Zheng et al. 2011). Interestingly, there was a strong correlation of nuclear AR positivity with nuclear β-catenin expression ($P=0.0215$), but not with membranous ($P=0.1781$) or cytoplasmic ($P=1.0000$) β-catenin expression.

To assess possible associations between β-catenin staining and disease progression, we then performed the Kaplan–Meier analysis coupled with log-rank test. Of the 24 patients, eight (33.3%) developed local recurrence and/or metastasis after radical surgery. There was a strong association of reduced membranous expression ($P=0.0276$; Fig. 1E), but not of cytoplasmic expression ($P=0.7532$; Fig. 1F), with poorer prognosis. Furthermore, nuclear β-catenin expression alone ($P=0.0802$; Fig. 1G) or co-expression of nuclear AR and β-catenin ($P=0.0043$; Fig. 1H) strongly correlated with tumor progression after cystectomy. Based on these findings, we anticipated that cross talk between AR and β-catenin signals contributed to the progression of bladder cancer.

Androgen upregulates the expression of active-β-catenin

We then investigated the effects of androgens on the expression of β-catenin in bladder cancer cells. Western blotting was performed in the stable cell lines with or without AR (i.e. UMUC3-control-shRNA vs UMUC3-AR-shRNA, 5637-AR vs 5637-V, and J82-AR vs J82-V) in the presence or absence of a synthetic androgen R1881 and an AR antagonist HF. Each cell line was found to strongly express β-catenin, and no significant differences in total β-catenin expression among the different treatment groups were observed (Fig. 2A). In contrast, R1881 considerably increased the expression of both an active form of β-catenin and its downstream target c-myc only in AR-positive cells. As expected, HF showing marginal or partial agonist activity could, at least partially, abolish the effects of R1881. A quantitative RT-PCR was also performed to determine whether androgen alters β-catenin and c-MYC gene expression in these cell lines. Correlating with the expression of c-MYC protein, R1881 increased its mRNA levels by 59%/57%/38% in UMUC3-control-shRNA/5637-AR/J82-AR respectively but not in AR-negative lines (Fig. 2B). HF significantly antagonized the effects of R1881 on c-MYC expression in AR-positive cells. Additionally, treatment with R1881 and/or HF resulted in marginal changes in total β-catenin mRNA expression. These results suggest that not only androgens may be able to activate β-catenin in bladder cancer cells but also AR is likely necessary for androgenic upregulation of active-β-catenin and c-MYC expression.

Androgen enhances β-catenin/TCF/LEF1 transcriptional activity

To further confirm whether Wnt/β-catenin signaling is activated by androgens in bladder cancer cells,
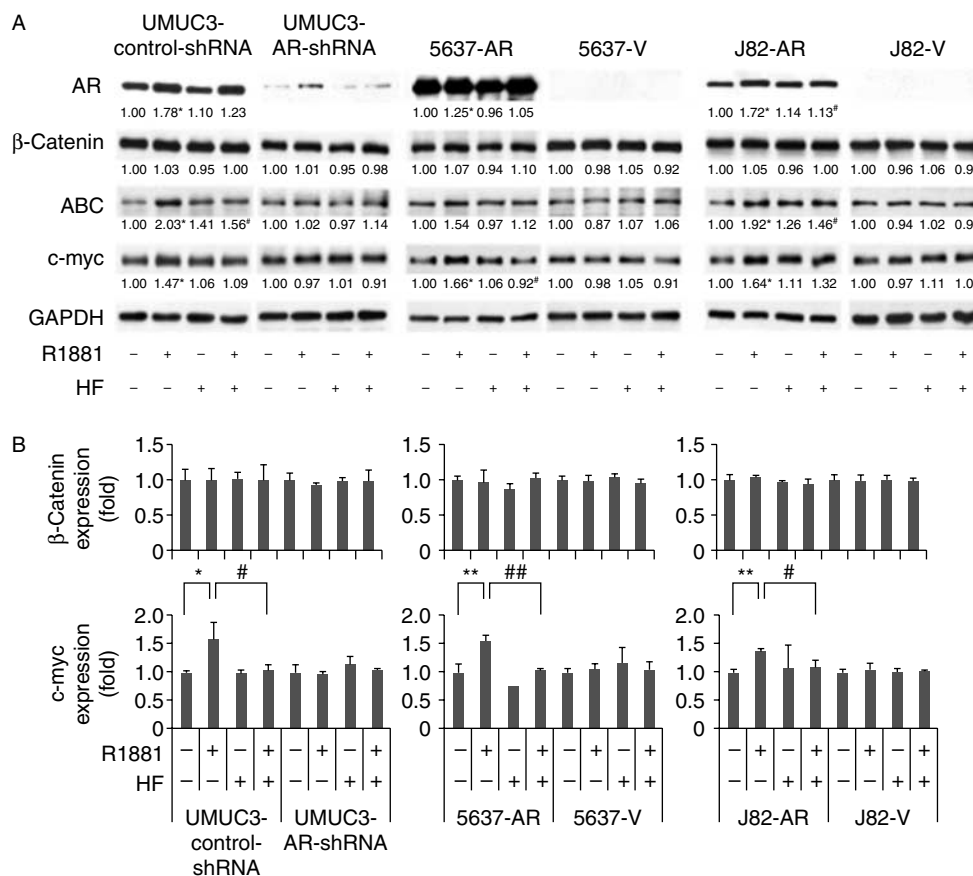
Endocrine-Related Cancer

Figure 2

Effects of androgen and antiandrogen on β-catenin expression in bladder cancer cells. Bladder cancer lines (UMUC3-control-shRNA/AR-shRNA, 5637-AR/Vector, and J82-AR/Vector) cultured for 24 h in the presence of ethanol, 10 nM R1881, and/or 10 μM HF were analyzed on western blotting (A) and quantitative RT-PCR (B). (A) Equal amounts of protein (30–50 μg) extracted from each cell line were immunoblotted for AR (110 kDa), β-catenin (92 kDa), active-β-catenin (ABC, 92 kDa), and c-myc (57 kDa). GAPDH (37 kDa) served as an internal control. Densitometry values for specific bands standardized by GAPDH that are relative to those of mock treatment (first lanes; set as onefold) from at least three independent experiments are included below the lanes. *$P<0.05$ (vs mock treatment). #$P<0.05$ (vs R1881 only). (B) RNA extraction and subsequent real-time RT-PCR for β-catenin and c-MYC were performed, and the expression of each gene was normalized to that of GAPDH. Transcription amount is presented relative to that of mock treatment in each cell line (first lanes; set as onefold). Each value represents an average and s.d. from at least three independent experiments. *$P<0.05$ (vs mock treatment). **$P<0.01$ (vs mock treatment). #$P<0.05$ (vs R1881 only). ##$P<0.01$ (vs R1881 only).

luciferase reporter assays were performed in the cell extracts with transfection of a plasmid harboring optimal TCF/LEF binding sites (Topflash) or mutated TCF/LEF binding sites (Fopflash) as a negative control and treatment of R1881 and/or HF. R1881 increased Topflash/Fopflash activity in AR-positive cells (UMUC3-control-shRNA (1.23-fold; $P<0.01$), 5637-AR (1.65-fold; $P<0.05$), and J82-AR (2.73-fold; $P<0.01$)), compared with respective mock treatments (Fig. 3). HF showing marginal agonist activity could block R1881-induced transactivation in these lines (all $P<0.05$). Conversely, in AR knockdown or negative lines, R1881 and HF failed to impact the activity of β-catenin/TCF/LEF1 transcription. Thus, these results suggest that androgen upregulates

Wnt/β-catenin signaling via the AR pathway in bladder cancer cells.

Androgen induces nuclear translocation of β-catenin and its co-localization with AR

To investigate whether AR signals promote nuclear translocation of β-catenin in bladder cancer cells, as shown in prostate cancer and neuronal cells (Mulholland et al. 2002, Pawlowski et al. 2002, Yang et al. 2002), western blotting was first performed using nuclear and cytoplasmic fractions obtained from AR-positive 5637-AR or UMUC3 cells cultured with different concentrations of R1881 for 24 h. Accumulations of nuclear β-catenin were seen upon
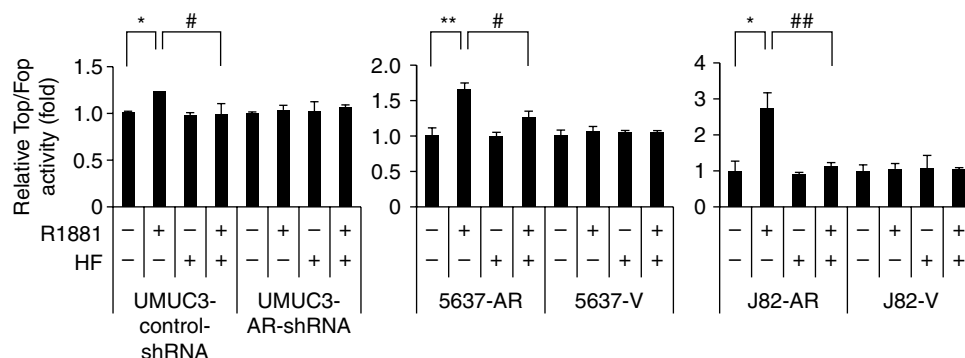
Figure 3

Effects of androgen and antiandrogen on β-catenin transactivation in bladder cancer cells. Bladder cancer lines (UMUC3-control-shRNA/AR-shRNA, 5637-AR/Vector, and J82-AR/Vector) co-transfected with a Topflash or Fopflash luciferase reporter plasmid were cultured for 24 h in the presence of ethanol, 10 nM R1881, and/or 10 μM HF. Luciferase activity analyzed in a luminometer is presented relative to that of mock treatment in each cell line (first lanes; set as onefold). Each value represents an average and s.D. from at least three independent experiments. *$P < 0.05$ (vs mock treatment). **$P < 0.01$ (vs mock treatment). #$P < 0.05$ (vs R1881 only). ##$P < 0.01$ (vs R1881 only).

R1881 treatment in a dose-dependent manner, although decreases in cytoplasmic β-catenin were mostly modest (Fig. 4A). HF antagonized the effect of R1881 on nuclear expression of β-catenin in UMUC3 cells (Fig. 4B).

To further assess androgen-induced nuclear translocation of β-catenin and its co-localization with AR, we performed immunofluorescent staining in three bladder cancer cell lines. As is well known in non-bladder cells, non-ligand-bound AR predominantly expressed in the cytoplasm was translocated into the nucleus of 5637-AR, J82-AR, and UMUC3-control-shRNA cells in the presence of R1881 (Fig. 4C). Unlike neuronal cells, β-catenin was predominantly distributed in the membrane and cytoplasm of bladder cancer cells in the absence of ligand, with roughly 10% in the nucleus (Fig. 4D). After R1881 treatment, nuclear β-catenin expression was seen in 20–42% of AR-positive cells, while membranous β-catenin staining was not significantly altered. Importantly, there were slight decreases in cytoplasmic β-catenin staining in R1881-treated cells compared with untreated cells. Consistent with previous studies in non-bladder cells (Mulholland et al. 2002, Pawlowski et al. 2002, Yang et al. 2002), co-localization of β-catenin and AR in the nucleus was observed in 57–72% of the R1881-treated cells with nuclear staining of β-catenin. By contrast, no R1881-induced nuclear β-catenin expression was seen in AR-negative or AR knockdown bladder cancer cells.

Androgen induces AR association with β-catenin and TCF

Having found evidence for co-localization of AR and β-catenin in the nuclei of bladder cancer cells, we finally performed co-immunoprecipitation to detect the physical interactions between AR and β-catenin or TCF. Protein extracts were immunoprecipitated with AR or β-catenin, and the immunoprecipitates were analyzed by immunoblotting using anti-β-catenin, anti-AR, or anti-TCF4 antibody (Fig. 5). In AR-positive cells, minimal interactions between AR and β-catenin, AR and TCF4, or β-catenin and TCF4 were seen in the absence of androgens, and R1881 treatment significantly enhanced these interactions. β-Catenin–TCF4 interaction was detected in AR-negative cells, but it was not altered by R1881 treatment. Thus, androgens appeared to induce complex formation among AR, β-catenin, and TCF4 in bladder cancer cells.

Discussion

We and others have documented that AR signals have stimulatory effects on bladder cancer cell proliferation (Miyamoto et al. 2007, Johnson et al. 2008, Boorjian et al. 2009, Wu et al. 2010, Zheng et al. 2011, Izumi et al. 2012). Dysregulation of the Wnt/β-catenin signaling pathway has also been linked to bladder cancer growth (Bui et al. 1998, Shiina et al. 2002, Hsieh et al. 2004, Stoehr et al. 2004, Urakami et al. 2006, Kastritis et al. 2009, Hirata et al. 2012). However, cross talk between the AR and Wnt/β-catenin pathways in bladder cancer cells remains unclear, although it has been well studied in prostate cancer (Chesire & Isaacs 2003, Wang et al. 2008). This study demonstrated molecular evidence for the involvement of AR signals in dysregulation of the Wnt/β-catenin pathway in bladder cancer cells. First, immunohistochemistry (IHC) in bladder
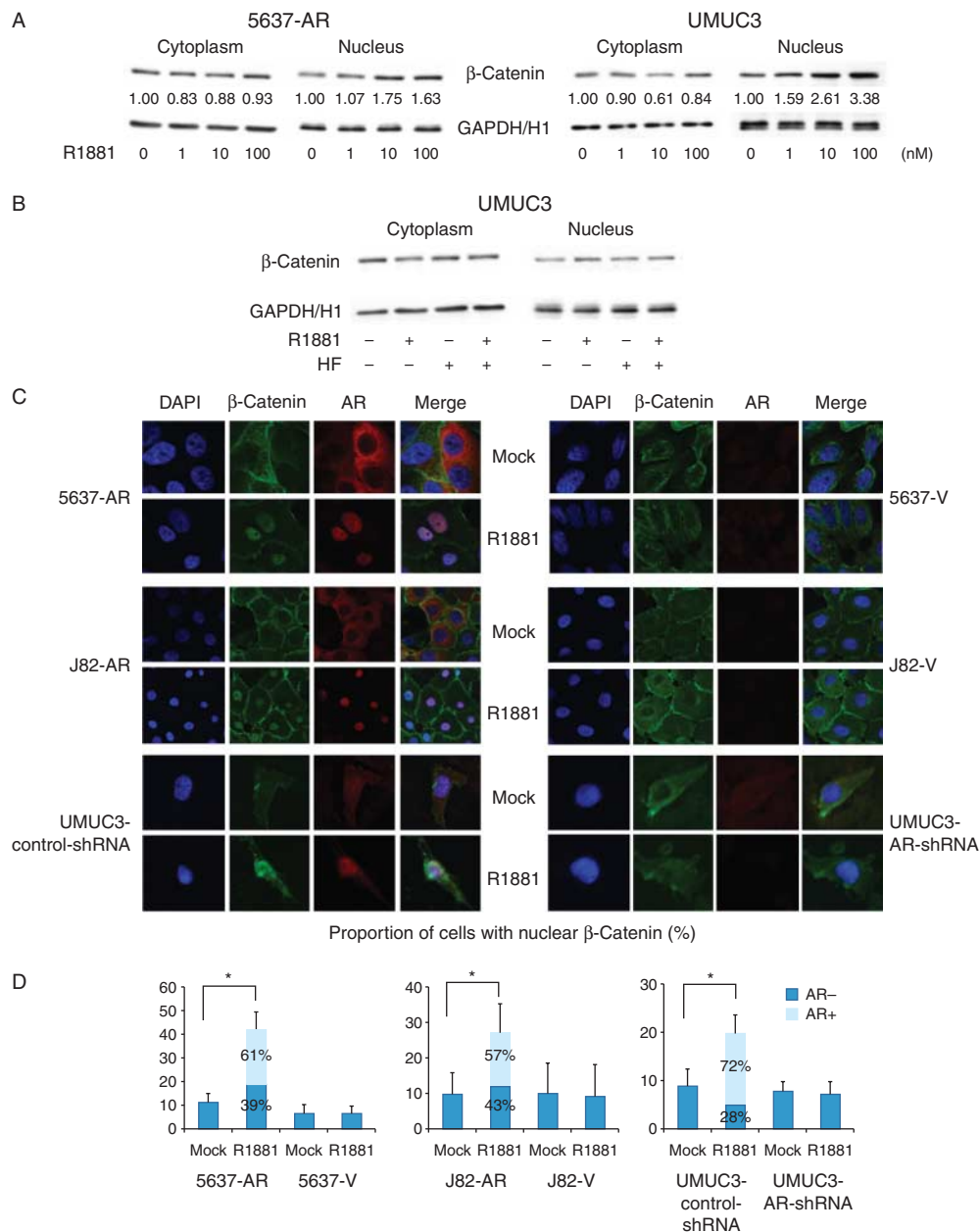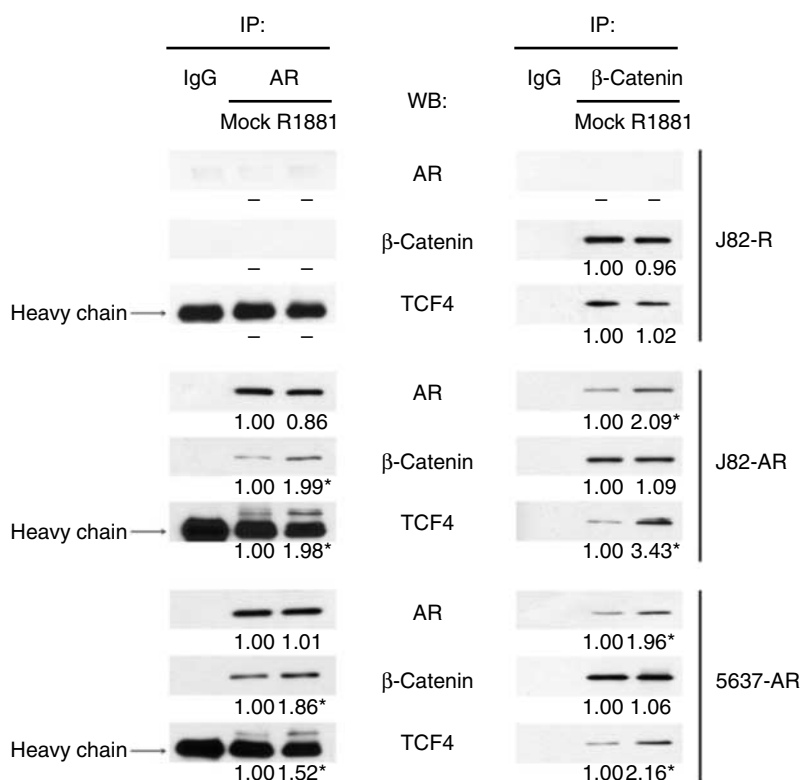
Figure 4

Effects of androgen on nuclear translocation of β-catenin and its co-localization with AR in bladder cancer cells. Cell lysates from 5637-AR or UMUC3 (A) cultured for 24 h in the presence of increasing amounts of R1881 or those from UMUC3 (B) cultured for 24 h in the presence of ethanol, 1 nM R1881, and/or 10 μM HF were fractionated into cytoplasmic and nuclear components and immunoblotted for β-catenin (92 kDa). GAPDH (37 kDa) and histone-H1 (32–33 kDa) served as internal controls for cytoplasmic and nuclear proteins respectively. Densitometry values for specific bands standardized by GAPDH or histone-H1 that are relative to those of mock treatment (first lanes; set as onefold) are included below the lanes. (C) Cells (5637-AR/Vector, J82-AR/Vector, UMUC3-control-shRNA/AR-shRNA) treated with ethanol (mock) or 10 nM R1881 for 24 h were analyzed on immunofluorescence, using an antibody to β-catenin or AR. DAPI was used to visualize nuclei. (D) Nuclear expression of β-catenin and AR was quantified. Each value represents an average and s.d. of triplicates. *P<0.01.

cancer tissues suggested a strong association between nuclear expression of AR and β-catenin, and their co-expression precisely predicted tumor progression. Secondly, in AR-positive bladder cancer cells, androgen likely activated β-catenin via increases in protein expression of its active form or a Wnt target c-myc, β-catenin/TCF/LEF1 transactivation, and nuclear translocation of AR and β-catenin. Finally, we showed androgen-mediated complex

Figure 5

Effects of androgen on AR/β-catenin/TCF4 associations. Cells (J82-Vector, J82-AR, 5637-AR) were cultured for 24 h in the presence of ethanol (mock) or 10 nM R1881. Cell lysates immunoprecipitated with anti-AR antibody/normal rabbit IgG or anti-β-catenin antibody/normal mouse IgG were then immunoblotted for rabbit anti-AR (110 kDa), mouse anti-β-catenin (92 kDa), or rabbit anti-TCF4 (66–72 kDa). Standardized densitometry values for specific blots, compared to mock treatment (set as onefold), from three independent experiments are included below the lanes. *$P < 0.05$ (vs mock treatment).

formation involving AR, β-catenin, and TCF4 in bladder cancer cells.

There are dissenting data as to the correlation of β-catenin staining in bladder cancer with tumor aggressiveness (Garcia del Muro *et al.* 2000, Nakopoulou *et al.* 2000, Zhu *et al.* 2000, Stoehr *et al.* 2002, Kastritis *et al.* 2009). The discrepancy may have resulted from the use of different antibodies and methodologies. Consistent with a previous study (Zhu *et al.* 2000), downregulation of membranous β-catenin expression in bladder cancer compared with non-neoplastic urothelium was detected in our cohort. Our current data also corroborated the demonstration that loss or reduced expression of membranous β-catenin was associated with worse outcome (Garcia del Muro *et al.* 2000, Nakopoulou *et al.* 2000). Nuclear accumulation of β-catenin, as a hallmark of Wnt/β-catenin activation (Chesire & Isaacs 2003), has been shown to correlate with lymph node involvement and poor prognosis (Kastritis *et al.* 2009). Consistent with these findings, our data showed a trend to associate

between nuclear β-catenin expression and a risk of tumor progression after cystectomy, while there was no relationship between lymph node metastases and the status of β-catenin expression in the nucleus as well as the membrane or cytoplasm, possibly due to relatively small number of cases. It was noteworthy that aberrant accumulation of nuclear β-catenin in conjunction with nuclear AR positivity was a more reliable poor prognosticator. AR expression was also closely correlated with the presence of nuclear β-catenin, but not membranous or cytoplasmic β-catenin, in bladder cancer tissues. Further IHC studies including larger patient cohorts with longer follow-up are needed to validate these preliminary findings of co-expression of AR and β-catenin in bladder cancer and its relationship with patients' outcomes. Nevertheless, the current data suggest that cross talk between the Wnt/β-catenin and AR pathways contributes to bladder cancer progression.

Our data in cultured cell lines may provide convincing evidence of the cross talk in bladder cancer. As seen in

prostate cancer (Chesire & Isaacs 2003, Wang *et al.* 2008), we anticipated that androgens regulated the expression of β-catenin and its nuclear translocation in bladder cancer cells, which could result in modification of β-catenin/TCF/LEF1 signaling and ultimately activate or inactivate target genes. Downstream components of the canonical Wnt/β-catenin signaling pathway, such as c-myc, cyclin-D1, and EGFR, have been implicated in several human malignancies including bladder cancer (Behrens *et al.* 1996, Brableltz *et al.* 2000, Tan *et al.* 2005), although the possibility remains that some of these are not direct targets *in vivo* and there are direct target genes relevant to bladder cancer. Western blots showed that androgen induced the expression of an active form of β-catenin, but not total β-catenin, only in AR-positive bladder cancer cells. Our β-catenin/TCF/LEF1 luciferase reporter assay then confirmed that androgen/AR enhanced β-catenin-mediated transactivation. Importantly, an antiandrogen HF could antagonize all these androgen effects in AR-positive bladder cancer cells. We also showed enhanced expression of c-myc at both mRNA and protein levels in androgen-treated cells. The *c-MYC* gene has indeed been found to correlate with the proliferation of bladder cancer cells (Lipponen 1995, Schmitz-Drager *et al.* 1997). Androgen/AR-mediated upregulation of other Wnt targets, including cyclin-D1 (Wu *et al.* 2010) and EGFR (Zheng *et al.* 2011), has also been demonstrated in bladder cancer cells. Furthermore, using immunofluorescence and western blotting, we validated AR-induced nuclear translocation of β-catenin, which has been investigated in several other types of cells (Mulholland *et al.* 2002, Pawlowski *et al.* 2002, Yang *et al.* 2002, Singh *et al.* 2006), in bladder cancer cells with endogenous or exogenous AR. Overall, available data suggest that AR activation positively modulates the Wnt/β-catenin pathway in bladder cancer cells.

It is well known that nuclear β-catenin is able to interact with not only TCF/LEF1 but also AR. Androgens have been shown to inhibit adipogenic differentiation (Singh *et al.* 2006) and promote myogenic differentiation (Singh *et al.* 2009) of mesenchymal multipotent cells through inducing AR association with β-catenin or TCF4. Consistent with these findings in non-bladder cells, our results indicate that AR activation induces nuclear accumulation of β-catenin, leading to interactions among β-catenin, TCF4, and AR. Surprisingly, weak associations of AR/β-catenin/TCF4 were detected without androgen treatment. There are several possibilities underlying this observation. First, remaining androgens in charcoal-stripped FBS (Sedelaar & Isaacs 2009) used for our cell culture led to AR activation since as low as 0.1 nM dihydrotestosterone was shown to regulate the growth of prostate cancer cell lines with endogenous or over-expressed AR (Mizokami *et al.* 2004, Waltering *et al.* 2009). Secondly, AR could be activated by non-androgenic compounds, such as growth factors (Culig *et al.* 1994). We recently found that EGF promoted the growth of bladder cancer cells via the AR pathway (Izumi *et al.* 2012). Thirdly, AR/β-catenin/TCF4/LEF1 might be able to form complexes in the cytoplasm. In some malignancies, substantial amounts of TCF4/LEF1 have been reported to localize to the cytoplasm (Shair *et al.* 2009, Tian *et al.* 2009). Our immunofluorescent staining showed co-localization of AR and β-catenin in the cytoplasm of some bladder cancer cells without adding androgens. Of note in the current study was that androgen further induced interactions of AR–β-catenin, AR–TCF4, and β-catenin–TCF4.

Despite the fact that the mechanism by which AR regulates β-catenin signaling has been elaborated in prostate cancer (Wang *et al.* 2008), some of the findings in AR-positive prostate vs bladder cancers were in disagreement. For instance, we observed upregulation, rather than downregulation, of β-catenin-mediated transcription by AR signals in bladder cancer. Our co-immunoprecipitation assays suggested physical interactions of not only AR–β-catenin but also AR–TCF4, in addition to β-catenin–TCF4 association, in bladder cancer cells, while in prostate cancer, competition for β-catenin could occur between AR and TCF/LEF1 (Mulholland *et al.* 2003). Mapping studies in non-bladder cells have indeed demonstrated that AR and TCF4 have overlapping binding sites on β-catenin and compete for binding (Yumoto *et al.* 2011). Taken together, our results form the basis of the following hypothetical model in bladder cancer cells. In the absence of androgens, AR and a portion of β-catenin are located in the cytoplasm, while β-catenin also resides in the membrane. Androgen-bound AR interacts with β-catenin and induces their nuclear translocation. In the nucleus, β-catenin-bound AR further interacts with TCF/LEF1 and thereby stimulates transcription of various Wnt/β-catenin target genes, leading to the promotion of bladder cancer cell growth. Mechanistic studies, such as mapping that may identify bladder-specific AR-binding sites, are required to further elucidate the role of β-catenin and TCF/LEF1 in relation to AR signals in bladder cancer progression.

In conclusion, we demonstrate, for the first time, that androgen activates Wnt/β-catenin signaling through the AR pathway in bladder cancer cells. Our data not only

suggest that androgen-induced β-catenin/TCF/LEF1 activity, possibly via formation of their complex involving AR, contributes to the regulation of bladder cancer progression in a specific manner but also provide further evidence enhancing the feasibility of androgen deprivation that may interfere with the complex formation as a potential therapeutic approach against bladder cancer.

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Expression of UDP-Glucuronosyltransferase 1A in Bladder Cancer: Association With Prognosis and Regulation by Estrogen

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Although UDP-glucuronosyltransferase 1A (UGT1A) plays an important role in preventing bladder cancer initiation by detoxifying carcinogenic compounds, its contribution to bladder cancer progression is poorly understood. We immunohistochemically stained for UGT1A in bladder specimens. UGT1A was positive in 130/145 (90%; 28 [19%] weak, 53 [37%] moderate, and 49 [34%] strong) urothelial neoplasms, which was significantly weaker than in matched non-neoplastic urothelial tissues (100/101 [99%]; 2 [2%] weak, 17 [17%] moderate, and 81 [80%] strong). Fifty (98%) of 51 low-grade/79 (99%) of 80 non-muscle-invasive tumors were immunoreactive to UGT1A, whereas 80 (85%) of 94 high-grade/51 (78%) of 65 muscle-invasive tumors were UGT1A-positive. Kaplan-Meier analysis showed strong associations between lower UGT1A expression versus the risk of recurrence in high-grade non-muscle-invasive tumors ($P = 0.038$) or disease-specific mortality in muscle-invasive tumors ($P = 0.016$). Multivariate analysis further revealed UGT1A loss as an independent prognosticator for disease-specific mortality in patients with muscle-invasive tumor ($P = 0.010$). Additionally, the expression of UGT1A was positively and negatively correlated with those of estrogen receptor-α and estrogen receptor-β, respectively. We then assessed UGT1A/Ugt1a levels in human cell lines/mouse tissues. 17β-Estradiol increased and decreased UGT1A expression in normal urothelium and bladder cancer lines, respectively, and an anti-estrogen abolished these effects. Ovariectomy in mice resulted in down-regulation of Ugt1a subtypes. These results suggest the involvement of UGT1A in not only bladder carcinogenesis but tumor progression. Moreover, UGT1A is likely regulated by estrogens in non-neoplastic urothelium versus bladder tumor in opposite manners, which could be underlying mechanisms of gender-specific differences in bladder cancer incidence and progression. © 2012 Wiley Periodicals, Inc.

Key words: 17β-estradiol; immunohistochemistry; ovariectomy; SVHUC cell line; urothelial neoplasm

INTRODUCTION

It is estimated that 73,510 individuals living in the United States will develop urinary bladder cancer in 2012 and 14,880 will die of the disease [1]. Data from comparative studies have demonstrated that bladder cancer affects men three to four times more often than women, while female patients tend to present with more aggressive tumor than male patients [1,2].

Bladder cancer is one of the first neoplasms recognized to be caused by exposure to carcinogenic compounds, such as industrial chemicals and cigarette smoke. Aromatic amines, well-known industrial bladder carcinogens, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a metabolite of the most potent and abundant procarcinogens derived from tobacco and its smoke, are glucuronidated in the liver and excreted into the urinary system [3,4]. It has been shown that UDP-glucuronosyltransferases (UGTs), belonging to the superfamily of major phase II drug metabolism enzymes, play a vital role in catalyzing the glucuronidation of carcinogens, including aromatic amines and NNAL [5].

Human UGTs are composed of UGT1A, UGT2A, and UGT2B, based on gene sequence homology [6]. In addition to the liver, the expression of UGTs has been detected in other organs, including the aerodigestive tract [7], gastrointestinal tract [8], and kidney [9]. It has also been reported that normal bladder expresses all the UGT subtypes except UGT2B17 [10] and that, compared with normal uro-

thelium, UGT1A expression is down-regulated in several bladder cancer tissue samples [11,12]. Recently, a genome-wide association study revealed a linkage between *UGT1A* gene locus and bladder cancer susceptibility [13]. Indeed, UGT1A, rather than the UGT2 family, has been suggested to contribute to metabolism of aromatic amines [5]. Down-regulation of Ugt1a, although there is no clear consensus about the functional homology between human UGT and mouse Ugt, was associated with higher incidence of chemically induced bladder cancer in mice [14]. Thus, UGT1A, consisting of nine functional proteins and four pseudogenes generated by alternative splicing, and Ugt1a, consisting of nine functional protein and five pseudogenes [15], are likely key enzymes involved in bladder carcinogenesis.

Although excessive exposure to industrial chemicals and cigarette smoke may have contributed to male dominance in bladder cancer, men remain at a substantially higher risk of bladder cancer than women even after controlling for these carcinogenic factors [16]. We have shown molecular evidence for this gender-specific difference by implicating androgen receptor (AR) signals in bladder cancer development [17]. There is also increasing evidence suggesting that other steroid hormone receptors are involved in bladder carcinogenesis and cancer progression [18]. Recently, we showed that dihydrotestosterone (DHT) reduced the expression of UGT1A subtypes via the AR pathway in normal urothelial cells [19]. In the previous report [19], we also showed that UGT1A was down-regulated in high-grade urothelial carcinoma tissues and strong expression of UGT1A correlated with favorable prognosis. However, the results were not conclusive presumably due to a relatively small number of cases ($n = 24$) including no low-grade tumors. The purpose of the current study is to validate the previous findings in larger patient cohorts with longer follow-up. We also assessed possible associations of UGT1A expression with AR and estrogen receptor (ER) signals in non-neoplastic urothelium and urothelial carcinoma.

MATERIALS AND METHODS

Tissue Samples

We retrieved 145 bladder tissue specimens obtained by transurethral resection or cystectomy performed at the University of Rochester Medical Center or the Johns Hopkins Hospital. All the sections were reviewed for confirmation of original diagnoses, according to the 2004 WHO/ISUP classification system for urothelial neoplasms [20], by two urologic pathologists (J.L.Y. and G.J.N.) at respective institutions. Appropriate approval from the Institutional Review Board at each institution was obtained prior to construction and use of the tissue microarray

(TMA). Bladder TMAs were constructed from formalin-fixed paraffin-embedded specimens (145 tumor tissues and 101 benign appearing tissues from bladders of patients with tumors), as previously described [21]. These patients included 110 men and 35 women, with a mean age of 66.0 yr (range: 30–89 yr) at the time of surgery and a mean follow-up of 31.6 months (range 2–164 months) after the surgery. The tumors included 11 papillary urothelial neoplasms of low malignant potential (PUNLMPs), 40 non-invasive (pTa) low-grade urothelial carcinomas, 29 non-muscle-invasive ($\leq$pT1) high-grade urothelial carcinomas, and 65 muscle-invasive ($\geq$pT2) high-grade urothelial carcinomas. All 65 patients with muscle-invasive tumor underwent cystectomy. None of the patients had received therapy with radiation or anticancer drugs preoperatively, except for 17 cases with intravesical bacillus Calmette-Guérin treatment prior to radical cystectomy. All of these 145 cases were included in our prior study analyzing 188 cases for the expression of AR, ERα, and ERβ [21].

Immunohistochemistry

Immunohistochemical staining was performed at the University of Rochester Medical Center, using the primary antibody to UGT1A (H300 clone; diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), as described previously [19,22]. An optimal condition for the stains was determined in control tissues. Each TMA contained orientation cores (tissues from multiple organs) that also served as internal positive and negative controls. All the stains were manually scored by one pathologist (H.M.) blinded to patient identity. German Immunoreactive Score (0–12) was calculated by multiplying the percentage of immunoreactive cells (0% = 0; 1–10% = 1; 11–50% = 2; 51–80% = 3; 81–100% = 4) by staining intensity (negative = 0; weak = 1; moderate = 2; strong = 3). Cores with the immunoreactive score of 0–1, 2–4, 6–8, and 9–12 were considered negative, weakly positive (1+), moderately positive (2+), and strongly positive (3+), respectively.

Cell Culture and Chemicals

Human urothelium cell line (SVHUC) and bladder cancer cell lines (UMUC3, 5637, and J82; all obtained from the American Type Culture Collection, Manassas, VA) were maintained in appropriate media (Mediatech, Manassas, VA; Kaighn's Modification of Ham's F-12 for SVHUC and Dulbecco's modified Eagle's medium for other cell lines) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. Cells were cultured in phenol-red free medium supplemented with 5% charcoal-stripped FBS at least 18 h before experimental treatment. We obtained DHT, 17β-estradiol (E2), and tamoxifen (TAM) from Sigma

(St. Louis, MO), and hydroxyflutamide (HF) from Schering (Kenilworth, NJ).

Western Blot

Protein extraction and Western blot were performed, as described previously [19,22] with minor modifications. Briefly, equal amounts of protein (20 μg) obtained from cell extracts were separated in 10% sodium dodecylsulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA) by electroblotting using a standard protocol. Specific binding of primary antibodies to ERα (E115 clone; diluted 1:1,000; Epitomics, Burlingame, CA), ERβ (14C8 clone; diluted 1:1,000; Abcam, Cambridge, MA), UGT1A (H300 clone; diluted 1:1,000), and GAPDH (6C5 clone; diluted 1:1,000; Santa Cruz Biotechnology) were detected, using horseradish peroxidase detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, IL).

Ovariectomized Mice

Female CL57BL/6-129SV mice were housed according to the institutional guidelines and were allowed food and water ad libitum. Mice received bilateral ovariectomy ($n = 9$) or sham surgery ($n = 6$) at 5 weeks of age. Among the mice undergoing oophorectomy, 0.1 ml of peanut oil with ($n = 3$) or without ($n = 6$) 20 μg of E2 was injected subcutaneously every 2 d. One week after the surgery, all the mice were sacrificed and urinary bladders were harvested. These specimens were rapidly frozen in liquid nitrogen and stored at $-80°C$ for subsequent RNA analysis.

Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction (PCR)

Total RNA (1.0 μg) isolated from harvested cell lines or mouse tissues, using TRIzol (Invitrogen, Carlsbad, CA), was reverse transcribed using 1 μmol/L oligo (dT) primers and four units of Omniscript reverse transcriptase (Qiagen, Valencia, CA) in a total volume of 20 μl. Real-time PCR was then performed in 15 μl system by using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously [19,22]. Primers used to amplify UGT1A mRNA were: 5′-TGATTGGTTTCCTCTTGGC-3′ and 3′-GGGTCTTGGATTTGTGGG-5′. The primer sequences for Ugt1a subtypes are given elsewhere [19]. Due to the high degree of homology, we were unable to design primers that separately amplify Ugt1a6a and Ugt1a6b.

Statistical Analyses

The Fisher's exact test was used to evaluate the association between categorized variables. Non-parametric two-group comparisons were carried out using Mann–Whitney U-test to assess differences in variables with ordered distribution across dichotomous categories. Survival rates in patients were calculated by the Kaplan-Meier method, and comparisons were made by log-rank test. These included comparisons among patients with non-invasive low-grade tumor (PUNLMP + low-grade carcinoma) with a mean follow-up of 37.1 months (range: 5–164), patients with non-muscle-invasive high-grade tumor with a mean follow-up of 26.8 months (range: 3–117), or patients with muscle-invasive tumor with a mean follow-up of 29.4 months (range: 2–141). Tumor recurrence was separately evaluated in 51 patients with non-invasive low-grade tumor and 29 patients with non-muscle-invasive high-grade tumor. Tumor progression was separately evaluated in 51 patients with non-invasive low-grade tumor (development of high-grade or invasive tumor), 29 patients with non-muscle-invasive high-grade tumor (development of muscle-invasive or metastatic tumor), and 65 patients with muscle-invasive tumor (development of local recurrence or metastatic tumor). Disease-specific survival was evaluated in 65 patients with muscle-invasive tumor. Correlation analyses were also performed, using the Spearman's correlation test. The Cox proportional hazards model was used to determine statistical significance of predictors in a multivariate setting. P values less than 0.05 were considered to be statistically significant.

RESULTS

Immunoreactivity in Benign and Carcinoma Tissues

We immunohistochemically investigated the expression of UGT1A in 145 bladder tumor specimens as well as corresponding 101 benign bladder tissues. Positive signals were detected predominantly in cytoplasms of non-neoplastic and neoplastic epithelial cells (Figure 1). Correlations of the expression status with different benign and tumor tissues are summarized in Table 1.

UGT1A was positive in 100 of 101 (99%; 2 [2%] 1+, 17 [17%] 2+, and 81 [80%] 3+) benign urothelial tissues and 130 of 145 (90%; 28 [19%] 1+, 53 [37%] 2+, and 49 [34%] 3+) urothelial neoplasms. Overall, UGT1A expression was significantly weaker in tumors than in benign tissues (score, $P < 0.001$).

Immunoreactivity and Clinicopathologic Features

We evaluated the correlation of expression levels of UGT1A stains with clinicopathologic features available for our patient cohort (Table 1). There were no statistically significant differences in UGT1A expression pattern in benign or neoplastic bladders between ages of the patients. Fifty (98%) of 51 low-grade tumors were immunoreactive to UGT1A (3 [6%] 1+, 25 [49%] 2+, and 22 [43%] 3+), and 80 (85%) of 94 high-grade tumors were UGT1A-positive (25 [27%] 1+, 28 [30%] 2+, and 27 [29%] 3). Thus, UGT1A expression was significantly lower
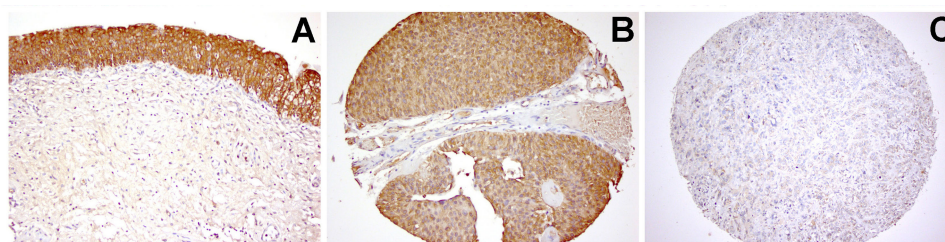
Figure 1. UGT1A immunohistochemisty in non-neoplastic urothelium (A) and bladder tumors (B and C). Strong stains for UGT1A were observed in non-neoplastic (A) and neoplastic (B) urothelial cells, whereas an invasive urothelial carcinoma (C) showed no immunoreactivity to UGT1A.

in high-grade carcinomas than in PUNLMPs + low-grade carcinomas (score, $P < 0.001$). Similarly, UGT1A expression was significantly lower in muscle-invasive tumors (51/65 [78%] positive; 21 [32%] 1+, 13 [20%] 2+, and 17 [26%] 3+) than in non-muscle-invasive tumors (79/80 [99%] positive; 7 [9%] 1+, 40 [50%] 2+, and 32 [40%] 3+; score, $P < 0.001$). However, among 68 cases with regional lymph node dissection, there was no significant difference in UGT1A levels between node-negative tumors (38/47 [81%] positive; 15 [32%] 1+, 14 [30%] 2+, and 9 [19%] 3+) and node-positive tumors (16/21 [76%] positive; 4 [19%] 1+, 5 [24%] 2+, and 7 [33%] 3+; score, $P = 0.607$).

We next performed Kaplan-Meier analysis coupled with log-rank test to assess possible associations of UGT1A staining with tumor recurrence or progression (Figure 2). For these analyses, we dichotomized UGT1A expression as 0/1+ vs. 2+/3+ in non-muscle-invasive tumors and 0 vs. 1+/2+/3+ in muscle-invasive tumors. Of the 51 patients with PUNLMP or low-grade carcinoma, 17 (33%) and 3 (6%) had recurrence and progression, respectively. There were no statistically significant correlations between UGT1A expression and recurrence ($P = 0.236$) or progression ($P = 0.683$). Of the 29 patients with non-muscle-invasive high-grade carcinoma, 14 (48%) and 7 (24%) had recurrence and progression, respectively. Low UGT1A levels were strongly associated with recurrence ($P = 0.038$), but not with progression ($P = 0.281$). Finally, of the 65 patients with muscle-invasive tumor, 35 (54%) had disease progression and 22 (34%) died of bladder cancer. Loss of UGT1A expression strongly correlated with disease-specific mortality ($P = 0.016$), but not with progression ($P = 0.168$).

To see whether UGT1A expression was an independent predictor of survival in patients with high-grade muscle-invasive tumor, multivariate analysis was performed with Cox model, including dichotomized pT stage (pT2 vs. pT3 + pT4), lymph node metastasis (pN0 vs. pN+), and UGT1A expression (0 vs. 1+/2+/3+; Table 2). In this subgroup, UGT1A expression was found to correlate with better cancer-specific survival (HR = 0.293; 95%CI = 0.116–0.745; $P = 0.010$), but not with tumor progression (HR = 0.574; 95%CI = 0.267–1.233; $P = 0.155$).

Association of UGT1A Expression With Gender or Expression of Sex Hormone Receptors

We investigated gender differences in UGT1A expression both in benign and tumor tissues (Table 3). UGT1A was positive in 73 of 74 (99%; 1 [1%] 1+, 14 [19%] 2+, and 58 [78%] 3+) male vs. 27 of 27 (100%; 1 [4%] 1+, 3 [11%] 2+, and 23 [85%] 3+) female benign tissues and 98 of 110 (89%; 19 [17%] 1+, 37 [34%] 2+, and 42 [38%] 3+) male vs. 32 of 35 (91%; 9 [26%] 1+, 16 [46%] 2+, and 7 [20%] 3+) female tumors. Although there were no statistically significant differences in UGT1A expression between males and females, its strong positivity (3+) was more often seen in male tumors than in female tumors ($P = 0.064$). Interestingly, these differences were more significant when separately analyzed in high-grade ($P = 0.006$), muscle-invasive ($P = 0.014$), or pN+ ($P = 0.046$; 0/1+ vs. 2+/3+) tumors, but not in low-grade ($P = 1.000$), non-muscle-invasive ($P = 0.606$), or pN0 ($P = 0.318$) tumors. However, no significant differences in UGT1A levels in either non-neoplastic or neoplastic bladders were seen between the two age groups of males only, females only, or all patients (e.g., ≤50 vs. ≥51 yr, ≤55 vs. ≥56 yr). In addition, in female patients, UGT1A expression still showed prognostic significance (i.e., recurrence of non-muscle-invasive high-grade tumor [0/1+ vs. 2+/3+, $P = 0.014$], progression [$P < 0.001$] or survival [$P = 0.055$] of muscle-invasive tumor [0 vs. 1+/2+/3+]; figures not shown). By contrast, in males, there were no statistically significant associations between UGT1A levels and patients' outcomes.

We then analyzed the correlations between expressions of UGT1A and AR/ERα/ERβ (Table 4). In our cohort of 101 benign and 145 malignant bladders where the expression of all these four proteins were examined, AR/ERα/ERβ was positive in 79 (78%)/54 (54%)/49 (49%) and 64 (44%)/38 (26%)/74 (51%), respectively. In benign tissues, there were no significant correlations between UGT1A and each

Table 1. Expression of UGT1A in Bladder Tissue Microarrays

	N	Positivity 0 (%)	1+ (%)	2+ (%)	3+ (%)	Fisher test (P value) 0 vs. 1+/2+/3+	0/1+ vs. 2+/3+	0/1+/2+ vs. 3+	Score (mean ± SD)	Mann–Whitney U-test (P value)
Tissue type										<0.001[a] / <0.001[b] / <0.001[c]
Benign urothelium (Age: mean ± SD, years)	101	1 (1) (64.00)	2 (2) (61.50 ± 0.71)	17 (17) (70.47 ± 10.81)	81 (80) (65.36 ± 12.64)	0.003[a] / 0.016[b] / 0.250[c]	<0.001[a] / <0.001[b] / 0.004[c]	<0.001[a] / <0.001[b] / <0.001[c]	2.81 ± 0.46	
						Not available[a,d]	0.589[a,d]	0.206[a,d]		
Urothelial neoplasm (Age: mean ± SD, years)	145	15 (10) (63.73 ± 10.38)	28 (19) (66.21 ± 10.61)	53 (37) (68.15 ± 12.02)	49 (34) (64.27 ± 14.39)	0.459[a,d]	0.682[a,d]	0.232[a,d]	2.01 ± 0.91	
Tumor grade						0.020[a,e] / 0.054[b,e] / 0.279[c,e]	<0.001[a,e] / 0.003[b,e] / 0.002[c,e]	0.099[a,e] / 0.680[b,e] / 0.006[c,e]		<0.001[a,e] / 0.013[b,e] / <0.001[c,e]
PUNLMP	11	1 (9)	1 (9)	7 (64)	2 (18)				2.09 ± 0.83	
Low-grade carcinoma	40	0 (0)	2 (5)	18 (45)	20 (50)				2.54 ± 0.47	
PUNLMP + Low grade	51	1 (2)	3 (6)	25 (49)	22 (43)				2.44 ± 0.59	
High-grade carcinoma	94	14 (15)	25 (27)	28 (30)	27 (29)				1.77 ± 0.97	
Tumor stage						<0.001[a,f] / 0.001[b,f] / 0.056[c,f]	<0.001[a,f] / <0.001[b,f] / <0.001[c,f]	0.112[a,f] / 0.431[b,f] / 0.027[c,f]		<0.001[a,f] / <0.001[b,f] / <0.001[c,f]
pTa	75	1 (1)	7 (9)	37 (49)	30 (40)				2.40 ± 0.59	
pT1	5	0 (0)	0 (0)	3 (60)	2 (40)				2.43 ± 0.44	
Non-muscle-invasive	80	1 (1)	7 (9)	40 (50)	32 (40)				2.40 ± 0.58	
pT2	23	5 (22)	9 (39)	3 (13)	6 (26)				1.50 ± 0.97	
pT3	31	7 (23)	10 (32)	7 (23)	7 (23)				1.46 ± 1.04	
pT4	11	2 (18)	2 (18)	3 (27)	4 (36)				1.79 ± 1.11	
Muscle-invasive	65	14 (22)	21 (32)	13 (20)	17 (26)				1.53 ± 1.01	
Lymph node involvement						0.749[a] / 0.708[b] / 1.000[c]	0.605[a] / 0.224[b] / 1.000[c]	0.228[a] / 0.102[b] / 1.000[c]		0.607[a] / 0.377[b] / 0.829[c]
pN0	47	9 (19)	15 (32)	14 (30)	9 (19)				1.52 ± 0.96	
pN+	21	5 (24)	4 (19)	5 (24)	7 (33)				1.66 ± 1.06	

[a] All cases.
[b] Males only.
[c] Females only.
[d] Student's t-test.
[e] PUNLMP + Low-grade carcinoma vs. High-grade carcinoma.
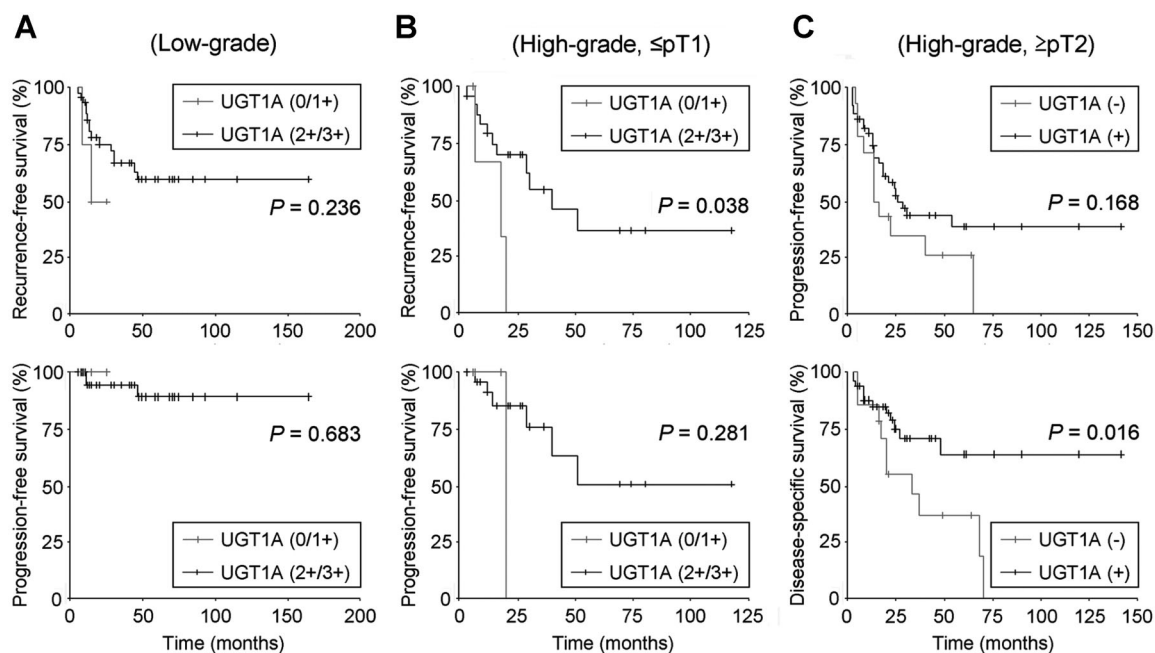[f] Non-muscle-invasive (pTa + pT1) vs. Muscle-invasive (≥pT2).

Figure 2. Kaplan-Meier analysis according to the levels of UGT1A expression. Recurrence/progression-free survivals in PUNLMPs and low-grade carcinomas (A) or non-muscle-invasive high-grade carcinomas (B) and progression-free/disease-specific survivals in muscle-invasive tumors (C). Comparisons were made by log-rank test.

hormone receptor. In neoplastic tissues, UGT1A showed a weak positive correlation ($0.2 < CC < 0.4$) with ERα and a weak negative correlation ($-0.4 < CC < -0.2$) with ERβ. When analyzed separately in men and women, these correlations were more significant in women (i.e., moderate positive correlation [$0.4 < CC < 0.7$] with ERα and moderate negative correlation [$-0.7 < CC < -0.4$] with ERβ).

Regulation of UGT1A/Ugt1a Expression by Sex Hormones

We assessed the effects of estrogen and androgen on UGT1A expression in human bladder cell lines. UMUC3 urothelial carcinoma line was shown to be AR-positive, whereas AR was undetectable in other

Table 2. Multivariate Cox Model in High-Grade Muscle-Invasive Tumors

	HR	95%CI	P value
Tumor progression			
pT[a]	3.338	1.337–8.334	0.010
pN[b]	1.490	0.718–3.090	0.284
UGT1A[c]	0.574	0.267–1.233	0.155
Cancer-specific mortality			
pT[a]	4.160	1.182–14.639	0.026
pN[b]	1.045	0.398–2.740	0.929
UGT1A[c]	0.293	0.116–0.745	0.010

[a]pT2 vs. pT3/pT4.
[b]pN0 vs. pN+.
[c]0 vs. 1+/2+/3+.

bladder cancer lines examined (5637, J82) and in SVHUC normal urothelial line [17,19,22]. We also examined the expression of ERα and ERβ in these cell lines. As shown in Figure 3A, all these lines expressed the ERβ, but not ERα. We then compared the levels of UGT1A protein expression in these cells treated with estrogen (E2) ± ER antagonist (TAM) or androgen (DHT) ± AR antagonist (HF). E2 clearly induced UGT1A expression in SVHUC, and TAM showing a partial agonist activity at least partially restored the E2 effect (Figure 3B). In contrast, E2 reduced UGT1A expression in three cancer cell lines, and TAM showing marginal agonist activities antagonized the E2 effect. We have shown that DHT treatment in SVHUC stably expressing AR resulted in decreases in UGT1A expression and HF antagonized the DHT effect [19]. However, DHT showed only marginal effects on UGT1A in UMUC3 (Figure 3C). We also tested mRNA expression of UGT1A in these cell lines treated with E2 or DHT by a quantitative real-time RT-PCR method. As expected, similar changes in UGT1A levels were observed (Figure 3D). These findings suggest that ERβ signals induce and repress UGT1A expression in normal urothelium and bladder cancer cells, respectively.

We found that female mice have higher levels of Ugt1a in their bladders, compared with male mice [19]. To further confirm the stimulatory effect of E2 on UGT1A expression in SVHUC, we evaluated Ugt1a expression in the bladders from wild-type

Table 3. Gender Difference in UGT1A Expression

	N	Positivity				Fisher test (P value)			Score (mean ± SD)	Mann–Whitney U-test (P value)
		0 (%)	1+ (%)	2+ (%)	3+ (%)	0 vs. 1+/2+/3+	0/1+ vs. 2+/3+	0/1+/2+ vs. 3+		
Benign										
Male	74	1 (1)	1 (1)	14 (19)	58 (78)	1.000	1.000	0.578	2.79 ± 0.48	0.288
Female	27	0 (0)	1 (4)	3 (11)	23 (85)				2.85 ± 0.43	
Neoplasm										
Male	110	12 (11)	19 (17)	37 (34)	42 (38)	0.765	0.527	0.064	2.04 ± 0.92	0.267
Female	35	3 (9)	9 (26)	16 (46)	7 (20)				1.91 ± 0.87	
PUNLMP + Low-grade carcinoma										
Male	38	1 (3)	3 (8)	18 (47)	16 (42)	1.000	0.342	1.000	2.39 ± 0.64	0.461
Female	13	0 (0)	0 (0)	7 (54)	6 (46)				2.59 ± 0.39	
High-grade carcinoma										
Male	72	11 (15)	16 (22)	19 (26)	26 (36)	1.000	0.216	0.006	1.86 ± 1.00	0.078
Female	22	3 (14)	9 (41)	9 (41)	1 (5)				1.50 ± 0.83	
Non-muscle-invasive tumor										
Male	59	1 (2)	5 (8)	28 (47)	25 (42)	1.000	1.000	0.606	2.40 ± 0.60	0.947
Female	21	0 (0)	2 (10)	12 (57)	7 (33)				2.42 ± 0.53	
Muscle-invasive tumor										
Male	51	11 (22)	14 (27)	9 (18)	17 (33)	1.000	0.226	0.014	1.64 ± 1.07	0.133
Female	14	3 (21)	7 (50)	4 (29)	0 (0)				1.14 ± 0.71	
pN0 tumor										
Male	40	7 (18)	12 (30)	12 (30)	9 (23)	0.605	0.416	0.318	1.62 ± 0.99	0.132
Female	7	2 (29)	3 (43)	2 (29)	0 (0)				1.00 ± 0.64	
pN+ tumor										
Male	15	4 (27)	0 (0)	4 (27)	7 (47)	1.000	0.046	0.120	1.87 ± 1.12	0.092
Female	6	1 (17)	4 (67)	1 (17)	0 (0)				1.11 ± 0.71	

Table 4. Correlation of UGT1A Expression With AR, ERα, or ERβ

	N	AR		ERα		ERβ	
		CC	*P* value	CC	*P* value	CC	*P* value
Benign							
All cases	101	−0.088	0.379	0.078	0.438	0.076	0.452
Male	74	0.154	0.442	0.087	0.667	0.105	0.601
Female	27	−0.152	0.195	0.086	0.468	0.067	0.568
Tumor							
All cases	145	0.106	0.204	0.224	0.007	−0.292	<0.001
Male	110	0.092	0.339	0.165	0.085	−0.219	0.021
Female	35	0.178	0.307	0.412	0.014	−0.578	<0.001

CC, correlation coefficient.

DISCUSSION

female mice undergoing bilateral ovariectomy or sham surgery followed by E2 or mock treatment. As shown in Figure 4, ovariectomy down-regulated all the subtypes of Ugt1a (18–50% decrease), and E2 supplement at least partially restored the effects of ovariectomy.

UGTs are enzymes that contribute to detoxifying bladder carcinogens including aromatic amines and NNAL [5]. A recent genome-wide association study identified UGT1A as one of the susceptibility loci of bladder cancer [13]. It has also been reported, using mouse bladder cancer models, that a chemical carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) reduces Ugt1a expression and that knockout of Nfr2 (nuclear factor-like 2) results in decreased Ugt1a and increased incidence of BBN-induced bladder cancer [14]. Together with other findings demonstrating down-regulation of UGT1A in bladder cancer [11,12,19], UGT1A likely plays an important role in prevention of bladder carcinogenesis.

Down-regulation of UGT1A mRNA was first reported in 9 of 10 bladder tumor samples (complete loss in four tumors), compared with matched benign bladder tissues [11]. Then, the same group immunohistochemically stained for UGT1A in 19 bladder tumors and found 6 of the tumors, mostly high-grade and/or invasive, were virtually negative for UGT1A whereas benign tissues consistently expressed it [12]. In our previous study involving 24 high-grade urothelial carcinomas [19], we showed down-regulation of UGT1A in 13 (54%) tumors as well as an association of strong UGT1A staining with a lower progression rate. In the current study, we analyzed 145 bladder tumors that included PUNLMPs and low-grade carcinomas and found inverse correlations between UGT1A levels versus tumor grade or pT stage. We also showed that decreased UGT1A expression was strongly associated with progression in high-grade non-muscle-invasive tumors and disease-specific mortality in muscle-invasive tumors. In addition, patients with UGT1A-negative muscle-invasive tumor tended to have a risk of disease progression. Multivariate analysis further revealed that loss of UGT1A in muscle-invasive tumors was an independent prognosticator of disease-specific mortality. In contrast, no significant correlations were seen between UGT1A expression status and recurrence/progression of low-grade tumors or progression of high-grade non-muscle-invasive tumors, probably because only few of low-grade and/or non-muscle-invasive tumors were negative or weakly positive for UGT1A. Of note was that, as we recently showed in the identical 68 cases undergoing lymph node dissection [23], pN status was not a prognostic factor ($P = 0.284–0.929$) in our cohort. Our results thus suggest that UGT1A has protective effects on not only bladder cancer development but also tumor progression.

Epidemiological and clinical evidence indicates that men have a substantially higher risk of bladder cancer, whereas women with bladder cancer have less favorable prognosis [1,2]. Recent experimental data have suggested the involvement of AR and ER signaling pathways in bladder tumorigenesis and cancer progression and, therefore, urothelial carcinoma, like prostate and breast cancers, is considered as an endocrine-related neoplasm [18]. Nevertheless, no significant gender difference in the expression of AR, ERα, or ERβ in bladder tumors has been found [21,24–28]. In the current study, although there were no statistically significant differences in UGT1A expression between male versus female tissues (both benign and malignant bladders), strong positivity of UGT1A was more often detected in male tumors than in female tumors ($P = 0.064$). It was likely that UGT1A was considerably down-regulated in potentially aggressive tumors from females compared with male tumors, but UGT1A levels in low-grade and/or superficial tumors were
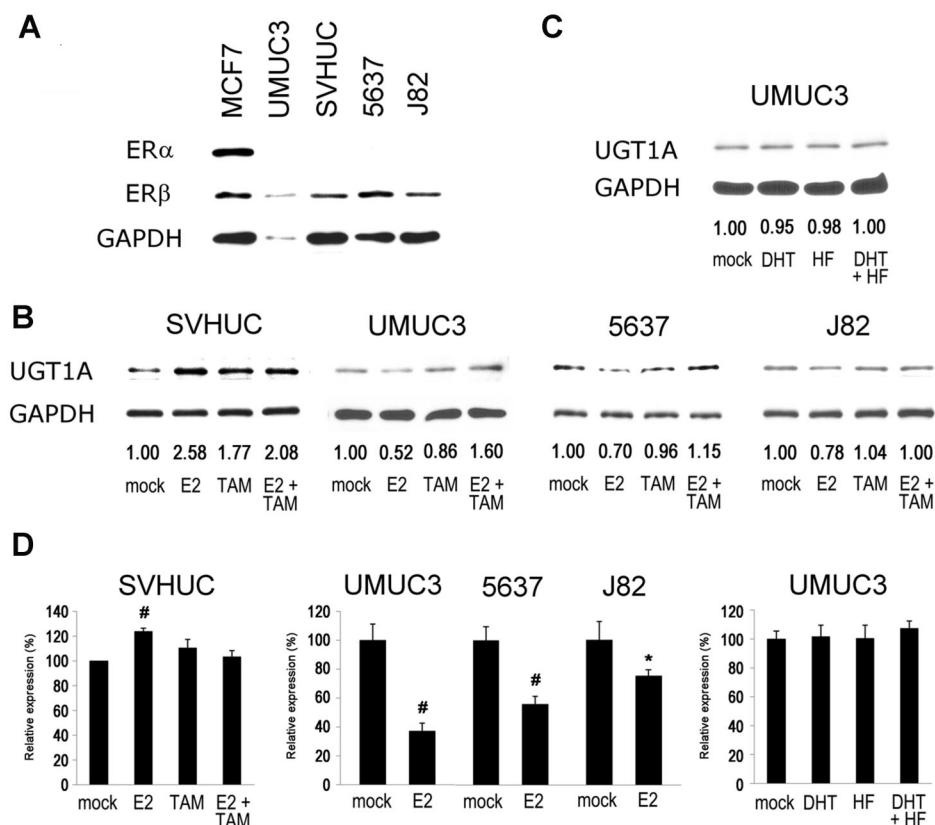
Figure 3. Regulation of UGT1A expression by sex steroids in benign and malignant urothelial cell liens. A: Protein extracts from SVHUC, UMUC3, 5637 and J82 cells were immunoblotted for ERα (66 kDa) and ERβ (56 kDa). MCF7 breast cancer line served as the positive control. B: Protein extracts from respective cell lines cultured in the presence of ethanol (mock), 1 nM E2, and/or 1 μM TAM for 24 h were immunoblotted for UGT1A (56 kDa). C: Protein extracts from UMUC3 cultured in the presence of ethanol (mock), 1 nM DHT, and/or 1 μM HF for 24 h were immunoblotted for UGT1A. In these western blots, GAPDH expression (37 kDa) served as the internal control. In (B) and (C), densitometry values for specific bands standardized by GAPDH that are relative to those of mock treatment (first lane in each cell line; set as onefold) are included below the lanes. Each value represents the mean from at least two independent experiments. D: Cell lines (SVHUC, UMUC3, 5637, and J82) cultured in the presence of ethanol (mock), 1 nM E2/DHT, and/or 1 μM TAM/HF for 24 h, as indicated, were analyzed on real-time RT-PCR for UGT1A. Expression of UGT1A was normalized to that of GAPDH. Transcription amount is presented relative to that of mock treatment in each line (first lane; set as 100%). Each value represents the mean + SD of triplicates. $^*P < 0.05$ (vs. mock treatment in the same cell line). $^\#P < 0.01$ (vs. mock treatment in the same cell line).

similar between genders of the patients. In addition, UGT1A tended to co-express with ERα and to express inversely with ERβ in bladder tumors, especially female tumors, whereas no significant correlation between expressions of UGT1A versus AR was found. In previous studies [21,25–27], expression levels of AR or ERα alone in bladder cancer exhibited no prognostic significance. However, some of these studies showed an association between higher ERβ expression and poorer patients' outcomes [21,27]. Furthermore, in a study using specific small interfering RNAs and selective agonists for ERα and ERβ, estrogens were shown to promote bladder cancer cell proliferation through the ER pathways [29]. Based on these findings, we hypothesized that in bladder cancer cells estrogens down-regulated UGT1A which may have a protective role in tumor progression. As expected, E2 repressed UGT1A expression at both mRNA and protein levels in all

three bladder cancer lines expressing ERβ, but not ERα, and TAM antagonized the E2 effect. Indeed, up to 76% of bladder tumors were reported to be positive for ERβ, while positive rates of ERα in immunohistochemical studies ranged from 1% to 27% [18,21,24,25,27,30,31]. In AR-positive UMUC3 cells, DHT did not alter the UGT1A level. Thus, estrogen-induced ERβ signals may promote bladder cancer progression via down-regulating UGT1A. Further analyses of UGTs in vitro and in vivo are necessary to determine its biological functions in bladder cancer growth, which may provide new insights into not only prognosis and progression but also novel therapeutic approaches.

We previously proposed that down-regulation of UGT1A by androgens in urothelial cells was a potential mechanism for male dominance in bladder cancer development [19]. We also showed significantly lower levels of Ugt1a in male mouse bladders, which
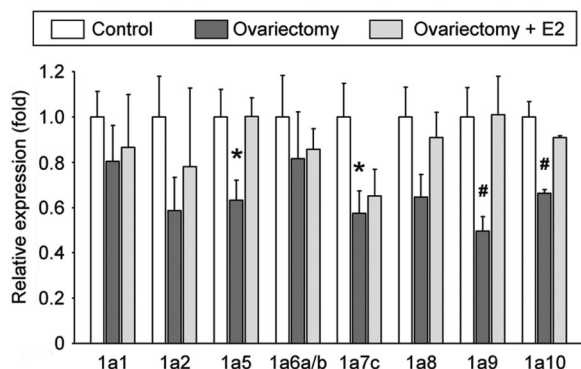
Figure 4. Effects of ovariectomy on Ugt1a expression in mouse bladder tissues. Wild-type female mice underwent sham surgery ($n = 6$), ovariectomy only ($n = 6$), or ovariectomy followed by E2 supplement ($n = 3$) at 5 weeks of age. One week after surgery, urinary bladders were harvested and analyzed on real-time RT-PCR for all the subtypes of Ugt1a. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that in wild-type females with sham surgery (first lanes; set as onefold). Each value represents the mean + SEM. *$P < 0.05$ (vs. control). #$P < 0.01$ (vs. control).

could be augmented by bilateral orchiectomy, than in those from females [19]. In addition, the levels of Ugt1a in castrated males or AR knockout males were still lower, compared with intact female mice. As shown in the liver [32], our previous results suggested the role of estrogen/ER signals in regulation of Ugt1a in the bladder. In the present study, we further demonstrated that bilateral ovariectomy led to decreases in Ugt1a expression in mouse bladders and estrogen supplement in ovariectomized mice restored the levels of Ugt1a. Consistent with these findings, E2 up-regulated the expression of UGT1A mRNA and protein in SVHUC normal urothelial cells expressing ERβ, but not ERα, which was at least partially abolished by an ER antagonist. Thus, it is likely that both androgens/AR and estrogens/ER signals are able to modulate UGT1A expression in the bladder, which may in turn affect the susceptibility to bladder carcinogens.

It is worth pointing out that bladder cancer is primarily a disease of advanced age. Thus, most of female bladder cancers are diagnosed after menopause in that serum levels of estrogens may not be different from those in male patients. Additionally, as aforementioned, studied have failed to show differential expression of ERs in bladder tumors between genders or ages of the patients [21,24,25,27]. These may not readily support important roles of estrogens and ER signals in bladder carcinogenesis and cancer progression. Although menopausal status of female patients was uncertain in our study, there was no significant difference in UGT1A levels in the younger cohorts compared with their older counterparts, possibly due to a relatively small number of women aged ≤50 ($n = 4$) or ≤55 ($n = 8$) yr.

However, it is still possible that high levels of UGT1A maintained by estrogens in women until menopause have reduced the exposure to bladder carcinogens excreted in the urine, which subsequently prevents or delays the development of bladder cancer even after menopause. We also showed that UGT1A levels, predominantly in female tumors, were associated with expression status of ERs and the prognosis. While estrogen levels in our cohort of patient were undetermined and might have varied, these results along with our in vitro data suggest the involvement of ER-mediated UGT1A signals in the growth of bladder cancer cells.

In conclusion, we showed down-regulation of UGT1A expression in bladder cancer and its inverse correlations with tumor grades and stages. Loss of UGT1A was an independent prognosticator for cancer-specific mortality in patients with muscle-invasive tumors. In addition, estrogen was found to up-/down-regulate UGT1A expression in normal urothelium/bladder cancer, respectively. These results not only suggest protective roles of UGT1A in both the development and progression of bladder cancer but may also provide potential underlying mechanisms responsible for gender-specific differences in the incidence and outcomes of bladder cancer. Further functional analyses of UGTs in bladder cancer are necessary to determine their biological significance.

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The Role of NFATc1 in Prostate Cancer Progression: Cyclosporine A and Tacrolimus Inhibit Cell Proliferation, Migration, and Invasion

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BACKGROUND. The functional role of nuclear factor of activated T-cells (NFAT), a well-characterized regulator of the immune response, in prostate cancer progression remains largely unknown. We aim to investigate biological significance of NFATc1, a NFAT isoform shown to function as an oncogene in a sarcoma model, in human prostate cancer.
METHODS. We first determined the expression levels of NFAT in prostate cell lines and tissue specimens. We then assessed the effects of NFAT inhibition via NFATc1-small interfering RNA (siRNA) as well as immunosuppressants including cyclosporine A (CsA) and tacrolimus (FK506) on prostate cancer cell proliferation, apoptosis, migration, and invasion in vitro and in vivo.
RESULTS. Immunohistochemistry revealed that the expression levels of NFATc1 were significantly elevated in prostatic carcinomas, compared with non-neoplastic prostate or high-grade prostatic intraepithelial neoplasia tissues, and in high-grade (Gleason scores ≥ 7) tumors. NFATc1 positivity in carcinomas, as an independent prognosticator, also correlated with the risk of biochemical recurrence after radical prostatectomy. In prostate cancer cell lines, CsA and FK506 inhibited NFATc1 expression and its nuclear translocation, NFAT transcriptional activity, and the expression of c-myc, a downstream target of NFAT. NFAT silencing or treatment with these NFAT inhibitors resulted in decreases in cell viability/colony formation and cell migration/invasion, as well as increases in apoptosis, in androgen receptor (AR)-negative, AR-positive/androgen-sensitive, and AR-positive/castration-resistant lines. No significant additional inhibition in the growth of NFAT-siRNA cells by CsA and FK506 was seen, whereas these agents, especially FK506, further inhibited their invasion. In xenograft-bearing mice, CsA and FK506 significantly retarded tumor growth.
CONCLUSIONS. Our results suggest that NFATc1 plays an important role in prostate cancer outgrowth. Thus, NFATc1 inactivation, especially using CsA and FK506, has the potential of being a therapeutic approach for not only hormone-naïve but also castration-resistant prostate cancers. *Prostate 75:573–584, 2015.* © 2015 Wiley Periodicals, Inc.

KEY WORDS: immunosuppressants; nuclear factor of activated T-cells; prostate cancer

INTRODUCTION

Cyclosporine A (CsA) and tacrolimus (FK506) are potent immunosuppressants widely used in organ transplant recipients to prevent rejection as well as occasionally prescribed in patients with autoimmune disorders [1,2]. It is well known that these agents specifically inhibit the nuclear factor of activated

T-cells (NFAT) pathway, leading to reduction of functional activity of not only T-cells but also other types of immune cells [1–4]. The NFAT family consists of five members: NFATc1 (also known as NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), NFATc4 (NFAT3), and NFAT5. These isoforms are located in the cytoplasm of immune cells and, upon cell stimulation in response to Ca^{2+}-calcineurin signals, translocate into the nucleus [5,6]. The nuclear NFATs then form heterodimers with other transcription factors and induce downstream gene transcription.

Emerging evidence has suggested that NFAT signaling plays an important role in tumorigenesis and tumor growth. In particular, as shown in immune cells, isoform-specific functions of NFAT in different types of neoplasms have been indicated. Among the isoforms, overexpression of NFATc1 has often been detected in, for instance, pancreatic [7], pulmonary [8], and hepatic [9] carcinomas, compared with their corresponding benign tissues. Of note, a constitutively active form of NFATc1 was shown to induce neoplastic transformation of fibroblast cells, suggesting its role as an oncogene, while NFATc2 was suggested to act as a tumor suppressor in the same study [10]. Further functional analyses have revealed that NFATc1 promotes tumor progression via induction of c-myc in pancreatic cancer [7], cyclooxygenase-2 in melanoma [11], and autotaxin/lysophosphatidic acid axis in breast cancer [12]. NFATc1 has also been implicated in lymphangiogenesis [13].

In prostate cancer, however, the involvement of specific NFAT isoforms in its growth has not been documented. Instead, intracellular calcium-mediated calcineurin signaling and subsequent activation of the NFAT pathway have been shown to promote the proliferation of LNCaP and primary culture cells representing androgen-dependent population of prostate cancer [14,15]. Interestingly, both CsA and FK506 suppressed androgen receptor (AR) activity as well as androgen-induced cell proliferation of LNCaP [16]. CsA could also retard the growth of PC3 and DU145 cells, but FK506 showed only marginal inhibitory effects in these AR-negative lines [16]. In addition, Cyp40 and FKBP51, immunophilins that CsA and FK506, respectively, bind, have been found to regulate androgen-mediated AR transactivation in prostate cancer cells [17]. It is also noteworthy that transplant recipients presumably receiving immunosuppressive medications have a significantly lower incidence of prostate cancer than expected, whereas immunosuppression increases the risks of subsequent development of most of other types of cancers [18]. Thus, NFAT signaling likely contributes to the development and cell proliferation of prostate cancer. In the current study, we investigated whether NFATc1 inactivation

and treatment with NFAT inhibitors lead to reduction in the proliferation/apoptosis of AR-negative, AR-positive/androgen-sensitive, and AR-positive/castration-resistant prostate cancer cells as well as in their migration and invasion ability.

MATERIALS AND METHODS

Prostate Tissue Microarray (TMA) and Immunohistochemistry

We retrieved 225 prostate tissue specimens obtained by radical prostatectomy performed at the University of Rochester Medical Center. Appropriate approval from the institutional review board was obtained before construction and use of the TMA. Prostate TMAs, consisting of representative lesions of benign, high-grade prostatic intraepithelial neoplasia (HGPIN), and prostatic adenocarcinoma, were constructed, as described previously [19]. None of the patients had received therapy with hormonal reagents, radiation, or other anti-cancer drugs pre- or post-operatively before clinical or biochemical recurrence. Biochemical recurrence was defined as a single prostate-specific antigen (PSA) level of $\geq 0.2\,ng/ml$.

Immunohistochemical staining was performed on the sections (5 µm thick) from the prostate TMAs or mouse xenograft tumors, as described previously [19,20]. Briefly, after deparaffinization, hydration, and antigen retrieval, samples were incubated overnight at 4°C with a primary antibody to NFATc1 (clone 7A6; dilution 1:50; Santa Cruz Biotechnology) or Ki-67 (clone 30–9; prediluted; Ventana) and then with a broad spectrum secondary antibody (Invitrogen). All stains were manually quantified by a single pathologist (H.M.) blinded to sample identify. For NFATc1 staining in the prostate TMAs, the German immunoreactive scores calculated by multiplying the percentage of immunoreactive cells (0% = 0; 1–10% = 1; 11–50% = 2; 51–80% = 3; 81–100% = 4) by staining intensity (negative = 0; weak = 1; moderate = 2; strong = 3) were considered negative (0; 0–1), weakly positive (1+; 2–4), moderately positive (2+; 6–8), and strongly positive (3+; 9–12).

Cell Culture and Chemicals

A human normal prostatic epithelium cell line (RWPE-1) and human prostate cancer cell lines (PC3, DU145, LNCaP, VCaP, C4–2, and CWR22Rv1) were maintained in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 and were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS at least 24 hr before experimental treatment. A small interfering RNA (siRNA) targeting

NFATc1 (sc-29412; Santa Cruz Biotechnology) or a non-silencing control-siRNA (sc-37007; Santa Cruz Biotechnology) was transfected at a final concentration of 20–80 nM into the prostate cancer cells, using GeneJuice (Novagen). We obtained CsA and FK506 from Abcam.

Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction (PCR)

Total RNA (0.5 μg) was isolated from cultured cells, using TRIzol (Invitrogen), and reverse transcribed with 1 μM oligo (dT) primers and 4 units of Omniscript reverse transcriptase (Qiagen) in a total volume of 20 μl. Semi-quantitative PCR and real-time PCR, using RT^2 SYBR® Green FAST Mastermix (Qiagen) for iCycler (Invitrogen), were then performed, as described previously [20–22]. The primer sequences are given in Table SI.

Western Blotting

Protein extraction and Western blotting were performed, as described previously [20–22] with minor modifications. We used a nuclear and cytoplasmic extraction reagent kit (NE-PER, ThermoScientific) for obtaining separate nuclear and cytoplasmic fractions. Equal amounts of protein (50 μg) obtained from cell extracts were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Immun-Blot PVDF Membrane, Bio-Rad) by electroblotting. Specific antibody binding was detected, using an anti-NFATc1 antibody (clone 7A6; dilution 1:500; Abcam) and a secondary antibody (mouse IRDye 680LT, LI-COR), followed by scanning with an infrared imaging system (Odyssey, LI-COR).

Reporter Gene Assay

Cells at a density of 50–70% confluence in 24-well plates were co-transfected with 250 ng of pGL4.30 NFAT reporter plasmid DNA (Promega) and 2.5 ng of pRL-TK plasmid DNA, using GeneJuice. After transfection, the cells were cultured in the presence or absence of CsA or FK506 for 24 hr. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit (Promega) and luminometer (FLUOstar Omega, BMG Labtech).

Cell Proliferation

We used the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay to assess cell viability, as described previously [20–22]. Briefly, cells $(1–3 \times 10^3)$ seeded in 96-well tissue culture plates were incubated in the presence or absence of CsA or FK506. The media were refreshed every 48 hr. After 96 hr of treatment, 10 μl MTT stock solution (5 mg/ml; Sigma) was added to each well with 100 μl of medium for 4 hr at 37°C. The medium was replaced with 100 μl dimethyl sulfoxide, followed by incubation for 5 min at room temperature. The absorbance was then measured at a wavelength of 570 nm with background subtraction at 655 nm using luminometer (FLUOstar Omega).

Colony Formation

Cells $(1 \times 10^3/\text{well})$ seeded in 6-well plates and cultured in the presence or absence of CsA or FK506 were allowed to grow until colonies in the control well were easily distinguishable. The cells were then fixed with methanol and stained with 0.1% crystal violet. The number of colonies and their areas were quantitated using ImageJ software (National Institutes of Health).

Apoptosis and Cell Cycle Analysis

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed on cell-burdening coverslips, using the DeadEnd Fluorometric TUNEL system (Promega), followed by counterstaining for DNA with 4′,6′-diamidino-2-phenylindole (DAPI), as described previously [21,22]. Apoptotic index was determined in the cells visualized by the fluorescence microscopy (EVOS FL Auto, Life Technologies). For cell cycle analysis, flow cytometry was performed in cells $(1 \times 10^6/10\,\text{cm dish})$ cultured in medium containing ethanol, CsA, or FK506 for 24 hr, harvested with trypsin, fixed in 70% ethanol, and stained with propidium iodide (PI) buffer (50 μg/ml; Life Technologies). Cellular PI content was measured on a Guava PCA-96 Base System™ flow cytometer (EMD Millipore) equipped with a green laser at 532 nm wavelength. Data were analyzed, using the Guava Cell Cycle software (EMD Millipore).

Cell Migration

In order to evaluate the ability of cell migration, a scratch wound healing assay was performed, as described previously [23]. Cells at a density of 90–100% confluence in 6-well plates were scratched manually with a sterile 200 μl plastic pipette tip, cultured in the presence or absence of CsA or FK506 for 24 hr, fixed with methanol, and stained with 0.1% crystal violet. The width of the wound area was quantitated, using ImageJ.

Cell Invasion

Cell invasiveness was determined, using a Matrigel (60 µg; BD Biosciences)-coated transwell chamber (8.0 µm pore size polycarbonate filter with 6.5 mm diameter; Corning), as described previously [21,23]. Briefly, cells (5×10^4) in 100 µl of serum-free medium were added to the upper chamber of the transwell, whereas 600 µl of medium containing 5% FBS was added to the lower chamber. The media in both chambers contained ethanol, CsA, or FK506. After incubation for 24 hr at 37°C in a CO_2 incubator, invaded cells were fixed, stained with 0.1% crystal violet, and counted under a light microscope.

Mouse Xenograft Model

Animal protocols in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals were approved at our institution. PC3 (5×10^5 cells/100 µl/site) resuspended in Matrigel (BD Biosciences) were subcutaneously injected into the flank of 6-week-old male immunocompromised NOD-SCID mice (Johns Hopkins Animal Resources). Treatment was initiated when the tumor volume reached 100 mm^3. Mice intraperitoneally received 30 mg/kg/day CsA, 3 mg/kg/day FK506, or vehicle control (corn oil) once-daily. Serial caliper measurements of perpendicular diameters were used to calculate tumor volume by the following formula: (short diameter)^2 × (longest diameter) × 0.5. After treatment, the mice were sacrificed for retrieval of the grafted tumors for immunohistochemistry.

Statistical Analysis

The Fisher exact test and the χ^2 test were used to evaluate the associations between categorized variables. The numerical data were compared by Student's t-test. Correlations between variables were determined by Spearman's correlation analysis. Survival rates in patients were calculated by the Kaplan–Meier method and comparison was made by log-rank test. The Cox proportional hazards model was used to determine statistical significance of predictors in a multivariate setting. P-values less than 0.05 were considered to be statistically significant.

RESULTS

Expression of NFATc1 in Human Prostate Cancer

We first examined the expression of NFAT in human prostate lines, including RWPE-1 normal cells as well as CWR22Rv1, LNCaP, VCaP, PC3, DU145,

and C4–2 cancer cells, by a semi-quantitative RT-PCR (Fig. 1A). These cell lines were found to express most of NFAT isoforms. Interestingly, NFATc1 expression was stronger in cancer cell lines (26–71% increases) than in RWPE-1 as well as in C4–2 (29% increase), an androgen-independent subline of LNCaP, than in the parental LNCaP. Moreover, NFATc2 expression was weaker in androgen-sensitive LNCaP and VCaP (74–85% decreases) and AR-negative PC3 and DU145 (32–52% decreases), compared with RWPE-1, but not in AR-positive/castration-resistant C4–2 (29% increase). The expression levels of NFATc3, NFATc4, and NFAT5 were similar among the cell lines examined, except VCaP showing their slightly weaker expression, compared with other lines.

We next stained immunohistochemically for NFATc1 in the prostate TMAs consisting of 225 radical prostatectomy cases. Positive signals were detected predominantly in the cytoplasm of epithelial cells (Fig. 1B). Overall, NFATc1 was positive in 91 of 225 (40.4%; 80 1+ and 11 2+) benign, 172 of 211 (81.5%; 102 1+, 67 2+, and 3 3+) HGPIN, and 196 of 225 (87.1%; 54 1+, 114 2+, and 28 3+) carcinoma tissues (Table I). Thus, NFATc1 expression was significantly stronger in carcinoma than in benign or HGPIN and in HGPIN than in benign. Significantly higher NFATc1 expression was also observed in Gleason score (GS) ≥7 tumors (vs. GS ≤6), but there were no statistically significant correlations between NFATc1 levels and tumor stage (pT/pN) or preoperative PSA levels [mean ± SD, 5.71 ± 2.69 in NFATc1(−); 5.99 ± 3.03 in NFATc1(1+); 5.91 ± 2.85 in NFATc1(2+); and 5.64 ± 2.17 in NFATc1(3+)]. Kaplan–Meier analysis coupled with log-rank test further revealed that patients with NFATc1-positive ($P = 0.034$) or NFATc1 (2+/3+) ($P = 0.003$) tumor had a significantly higher risk of biochemical recurrence after prostatectomy (Fig. 1C). To see whether NFATc1 expression was an independent predictor of tumor recurrence, multivariate analysis was performed with Cox model, including dichotomized GS, pathological stage, and NFATc1 expression (Table SII). In these subgroups, NFATc1 expression (0 vs. 1+/2+/3+, $P = 0.026$; 0/1+ vs. 2+/3+, $P = 0.018$) correlated with tumor recurrence.

Down-Regulation of NFAT by CsA and FK506 in Prostate Cancer Cells

We determined the effects of CsA and FK506, which were known to inactivate the NFAT pathway in immune cells [1–4], on NFATc1 expression by RT-PCR, Western blotting, and immunofluorescence in prostate cancer cells. NFAT-mediated transcriptional activity was also determined in the cell extracts with
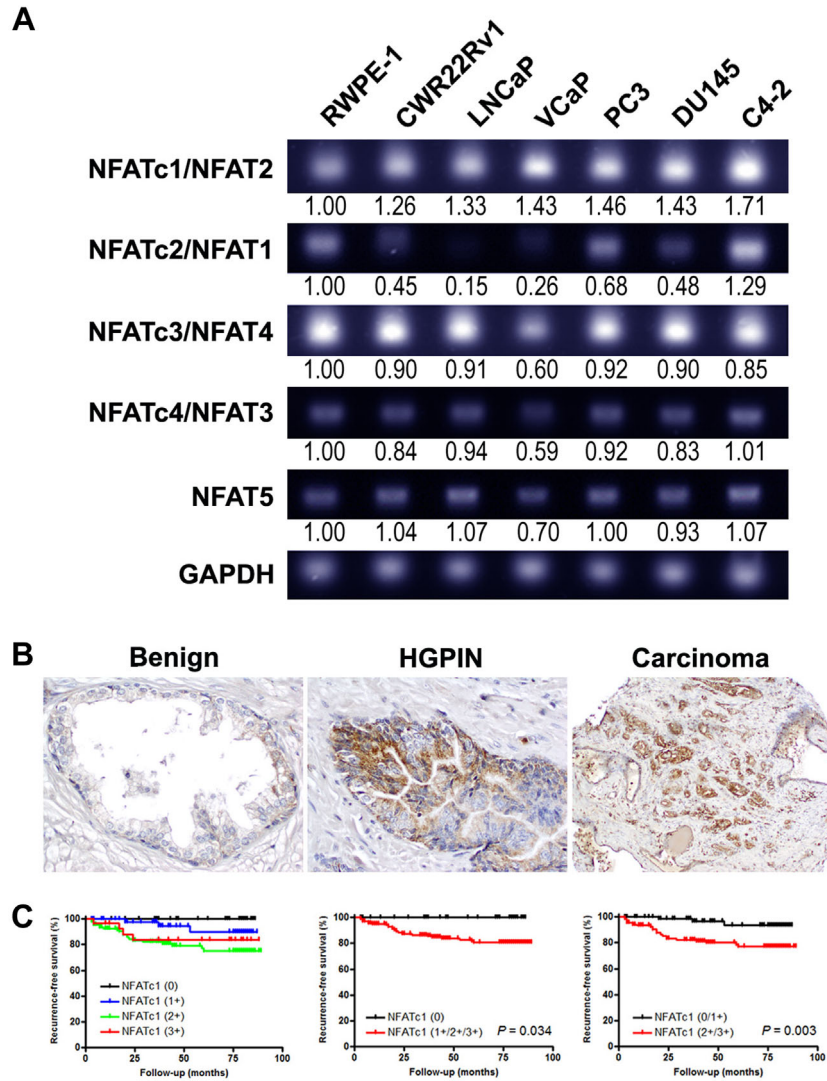
Fig. 1. Expression of NFATc1 in prostate cancer. **A**: Semi-quantitative RT-PCR of *NFATc1/NFAT2*, *NFATc2/NFAT1*, *NFATc3/NFAT4*, *NFATc4/NFAT3*, *NFAT5*, and *GAPDH* in prostate cell lines. Densitometry values for specific bands standardized by *GAPDH* that are relative to those in RWPE-1 cells are included below the lanes. **B**: Immunohistochemistry of NFATc1 in radical prostatectomy specimens. **C**: Recurrence-free survival rates in patients with prostate cancer. Kaplan–Meier analysis was performed according to the expression of NFATc1, and comparison was made by log-rank test.

transfection of a NFAT luciferase reporter plasmid. As expected, transfection of a NFATc1-siRNA silenced endogenous *NFATc1* mRNA in PC3 cells (Fig. 2A). Similarly, CsA and FK506 both at 1 µM reduced *NFATc1* gene expression in all prostate cancer cell lines tested (Fig. 2A). Subcellular localization of NFATc1 was then examined in PC3 by Western blotting: treatment with CsA and FK506 resulted in decreases in nuclear NFATc1 expression as well as a decrease (by CsA) or an increase (by FK506) in cytoplasmic NFATc1 expression (Fig. 2B). Prevention of nuclear translocation of NFATc1 was further confirmed by immunofluorescence (Fig. S1). Additionally,

NFATc1-siRNA (37% decrease) as well as CsA and FK506 (up to 69% decrease) reduced NFAT luciferase activity, compared with control-siRNA transfection or mock treatment (Fig. 2C). Less significant reduction in NFAT activity by the NFATc1-siRNA might be due to silencing of only one of NFAT isoforms. To confirm the down-regulation of NFAT activity by CsA and FK506, we measured the expression levels of c-myc, a downstream of NFAT signals [7]. Significant decreases in *c-myc* mRNA by NFATc1-siRNA and CsA/FK506 were seen (Fig. 2D). These results indicate that CsA and FK506 down-regulate the expression and activity of NFATc1 in prostate cancer cells.

TABLE I. Expression of NFATc1 in Prostate Tissue Microarrays

| | N | Negative | Positive | | | Score (mean ± SD) | P value | | | |
		0	1+	2+	3+		0 vs 1+/2+/3+	0/1+ vs 2+/3+	0/1+/2+ vs 3+	Score
Benign	225	134 (59.6%)	80 (35.6%)	11 (4.9%)	0 (0%)	1.74 ± 1.40	<0.001[a]	<0.001[a]	0.113[a]	<0.001[a]
HGPIN	211	39 (18.5%)	102 (48.3%)	67 (31.8%)	3 (1.4%)	3.72 ± 2.02	0.115[b]	<0.001[b]	<0.001[b]	<0.001[b]
Carcinoma	225	29 (12.9%)	54 (24.0%)	114 (50.7%)	28 (12.4%)	4.97 ± 2.53	<0.001[c]	<0.001[c]	<0.001[c]	<0.001[c]
Gleason score (GS)										
≤6	78	14 (17.9%)	22 (28.2%)	38 (48.7%)	4 (5.1%)	4.97 ± 2.54	0.142[d]	0.042[d]	0.018[d]	0.011[d]
7	134	13 (9.7%)	28 (20.9%)	69 (51.5%)	24 (17.9%)	5.35 ± 2.61	1.000[e]	0.557[e]	0.231[e]	0.609[e]
≥8	13	2 (15.4%)	4 (30.8%)	7 (53.8%)	0 (0%)	4.54 ± 2.18				
pT										
2	182	22 (12.1%)	48 (26.4%)	90 (49.5%)	22 (12.1%)	4.92 ± 2.51	0.614[f]	0.381[f]	0.797[f]	0.555[f]
3a	34	6 (17.6%)	4 (11.8%)	19 (55.9%)	5 (14.7%)	5.21 ± 2.62				
3b	9	1 (11.1%)	2 (22.2%)	5 (55.6%)	1 (11.1%)	5.06 ± 2.37				
pN										
0	112	15 (13.4%)	28 (25.0%)	54 (48.2%)	15 (13.4%)	4.96 ± 2.58	0.620	0.649	0.620	0.772
1	5	0 (0%)	1 (20.0%)	4 (80.0%)	0 (0%)	5.30 ± 1.47				

[a]Benign versus HGPIN.
[b]HGPIN versus Carcinoma.
[c]Benign versus Carcinoma.
[d]GS ≤6 versus GS ≥7.
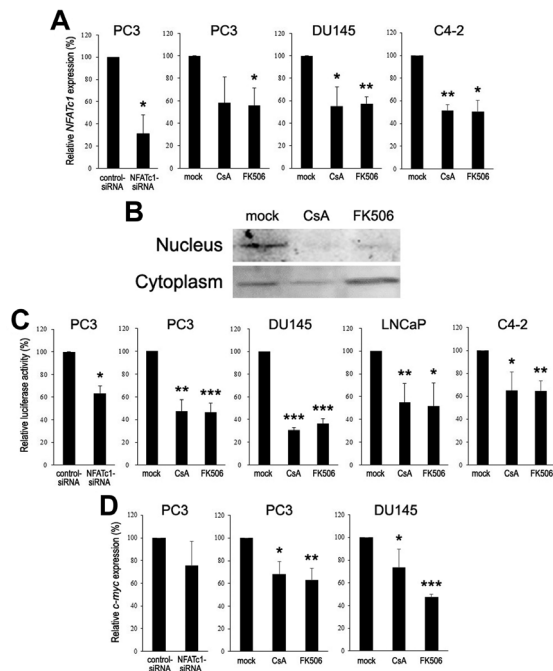[e]GS ≤7 versus GS ≥8.
[f]pT2 versus pT3.

Fig. 2. Inactivation of NFATc1 in prostate cancer. **A**: Expression of NFATc1 in prostate cancer cells. PC3 cells expressing control-siRNA or NFATc1-siRNA and original PC3/DU145/C4–2 cells treated with ethanol (mock), CsA (1 μM), or FK506 (1 μM) for 24 hr were subjected to real-time RT-PCR. Expression of *NFATc1* gene was normalized to that of *GAPDH*. Transcription amount is presented relative to that of control-siRNA expression or mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *$P < 0.05$ (vs. control-siRNA or mock treatment in each cell line). **$P < 0.01$ (vs. mock treatment in each cell line). **B**: Nuclear/cytoplasmic expression of NFATc1 in prostate cancer cells. Separate nuclear and cytoplasmic protein fractions from PC3 cells treated by ethanol (mock), CsA (1 μM), or FK506 (1 μM) for 24 hr were immunoblotted for NFATc1 (105 kDa). **C**: NFAT luciferase reporter activity in prostate cancer cells. PC3 cells with co-expression of control-siRNA or NFATc1-siRNA and original PC3/DU145/LNCaP/C4–2 cells were transfected with pGL4.30-NFAT-response element and pRL-TK and subsequently cultured with ethanol (mock), CsA (1 μM), or FK506 (1 μM) for 24 hr. Luciferase activity is presented relative to that of control-siRNA expression or mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *$P < 0.05$ (vs. mock treatment in each cell line). **$P < 0.01$ (vs. control-siRNA or mock treatment in each cell line). ***$P < 0.001$ (vs. mock treatment in each cell line). **D**: Expression of *c-myc* in prostate cancer cells. PC3 cells expressing control-siRNA or NFATc1-siRNA and original PC3/DU145 cells treated with ethanol (mock), CsA (1 μM), or FK506 (1 μM) for 24 hr were subjected to real-time RT-PCR. Expression of *c-myc* gene was normalized to that of *GAPDH*. Transcription amount is presented relative to that of control-siRNA expression or mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *$P < 0.05$ (vs. mock treatment in each cell line). **$P < 0.01$ (vs. mock treatment). ***$P < 0.001$ (vs. mock treatment).

Anti-Proliferative Effects of CsA and FK506 in Prostate Cancer Cells

To determine whether NFATc1 down-regulation exerts an influence on the proliferation of prostate cancer cells, we assessed cell viability (by MTT assay) and colony formation (by clonogenic assay) in those treated with CsA or FK506. In PC3, DU145, LNCaP, VCaP, and C4–2 cells, CsA and FK506 reduced their growth by 16–50% (Fig. 3A). Similar inhibition by CsA and FK506 was observed in PC3-control-siRNA cells (18–22% decreases). However, there were no significant additional decreases in the growth of PC3-NFATc1-siRNA cells, while an inhibitory effect of NFATc1-siRNA (17% decrease), compared with PC3-control-siRNA, was seen, suggesting the suppression by CsA or FK506 predominantly via NFATc1. In addition, both CsA and FK506 were found to significantly decrease the number and area of colonies in PC3, DU145, and C4–2 cells (Fig. 3B).

To investigate how CsA and FK506 inhibit cell proliferation, we performed TUNEL assay (Fig. 3C) and flow cytometry (Table SIII). CsA or FK506 treatment for 24 hr significantly increased apoptotic indices in PC3, DU145, and C4–2 cells. By contrast, these only marginally changed the G0/G1 population.

Suppressive Effects of CsA and FK506 on Prostate Cancer Cell Migration and Invasion

Cell migration and invasion are critical steps during tumor progression and metastasis. We therefore performed a scratch wound healing assay and a transwell invasion assay to assess the effects of NFATc1 inhibition on cell migration and invasion, respectively, in prostate cancer lines. In the wound healing assay, CsA and FK506 significantly delayed wound closure of PC3, DU145, LNCaP, and C4–2 (Fig. 4A). Similarly, in the transwell assay, CsA and FK506 treatment demonstrated marked decreases in cell invasion ability (Fig. 4B). The inhibitory effects of CsA and FK506 on the invasion appeared to be more significant in androgen-sensitive lines (72–87% decreases) than in AR-negative or AR-positive/castration-resistant lines (47–65% decreases). NFATc1 silencing also resulted in a significant decrease in the invasive properties (75% decrease), compared with the control line. In contrast to their effects on cell proliferation, CsA ($P = 0.112$) and FK506 ($P = 0.019$) appeared to further inhibit the invasion of PC3-NFATc1-siRNA cells, suggesting the involvement of the pathways other than NFATc1 in CsA/FK506-induced inhibition, although the efficacy of NFAT inhibition by CsA/FK506 in control cells (65–70% decreases) were similar to that of NFATc1 down-regulation alone. Using a quantitative RT-PCR
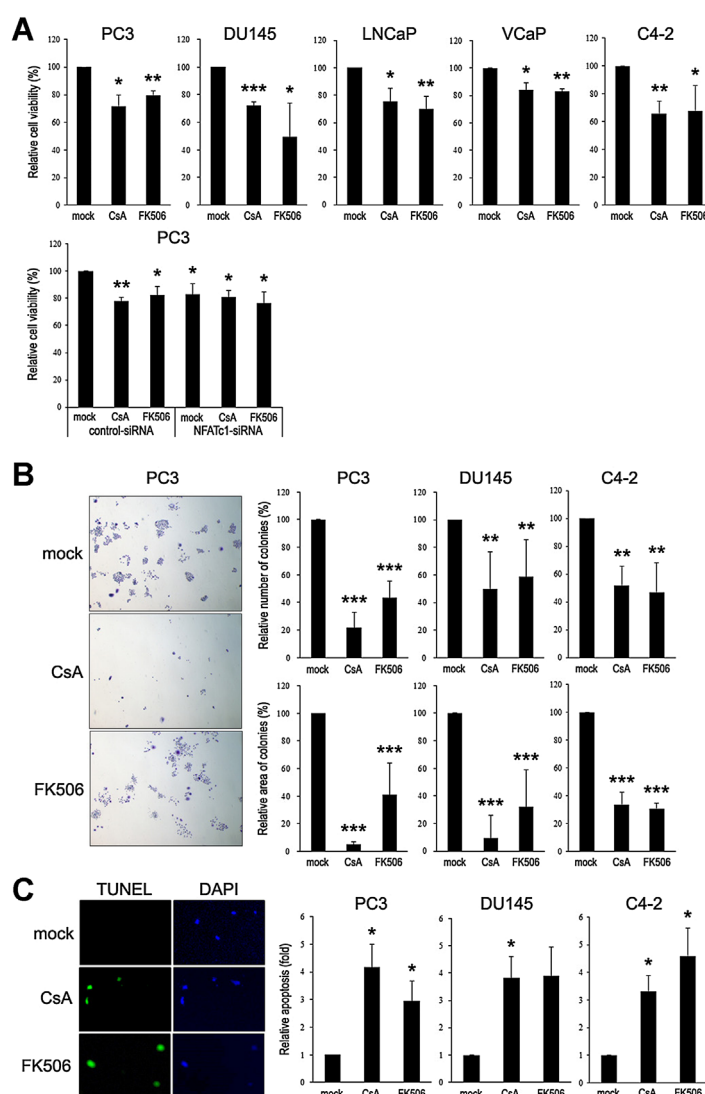
Fig. 3. Effects of NFAT inactivation on prostate cancer cell proliferation. **A**: MTT assay in parental PC3/DU145/LNCaP/VCaP/C4–2 cells and PC3 expressing control-siRNA or NFATc1-siRNA cultured with ethanol (mock), CsA (1 μM), or FK506 (1 μM) for 4 days. Growth suppression is presented relative to that of mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *$P < 0.05$ (vs. mock treatment in each cell line). **$P < 0.01$ (vs. mock treatment in each cell line). ***$P < 0.001$ (vs. mock treatment). **B**: Clonogenic assay in PC3/DU145/C4–2 cells cultured with ethanol (mock), CsA (1 μM), or FK506 (1 μM) for 2 weeks. The number of colonies and their areas quantitated using the ImageJ software are presented relative to those of mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *$P < 0.05$ (vs. mock treatment). **$P < 0.01$ (vs. mock treatment). ***$P < 0.001$ (vs. mock treatment in each cell line). **C**: TUNEL assay in PC3/DU145/C4–2 cells cultured with ethanol (mock), CsA (1 μM), or FK506 (1 μM) for 24 hr. Apoptosis (percentage of TUNEL-positive cells) is presented relative to that of mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *$P < 0.05$ (vs. mock treatment).

method, we then analyzed the effects of CsA and FK506 on the expression of MMPs that are known to play a critical role in cancer cell migration/invasion, angiogenesis, and resultant tumor progression and metastasis. NFATc1 knockdown as well as CsA and FK506 decreased the levels of *MMP-2* and *MMP-9* expression, compared with the control-siRNA or vehicle control, in four cell lines (Fig. 4C).

Anti-Tumor Activity of CsA and FK506 in a Mouse Xenograft Model for Prostate Cancer

Finally, we used a mouse xenograft model to investigate whether CsA and FK506 inhibit prostate tumor growth in vivo. PC3 cells were implanted subcutaneously into the flank of NOD-SCID mice, and, when the estimated tumor volume reached
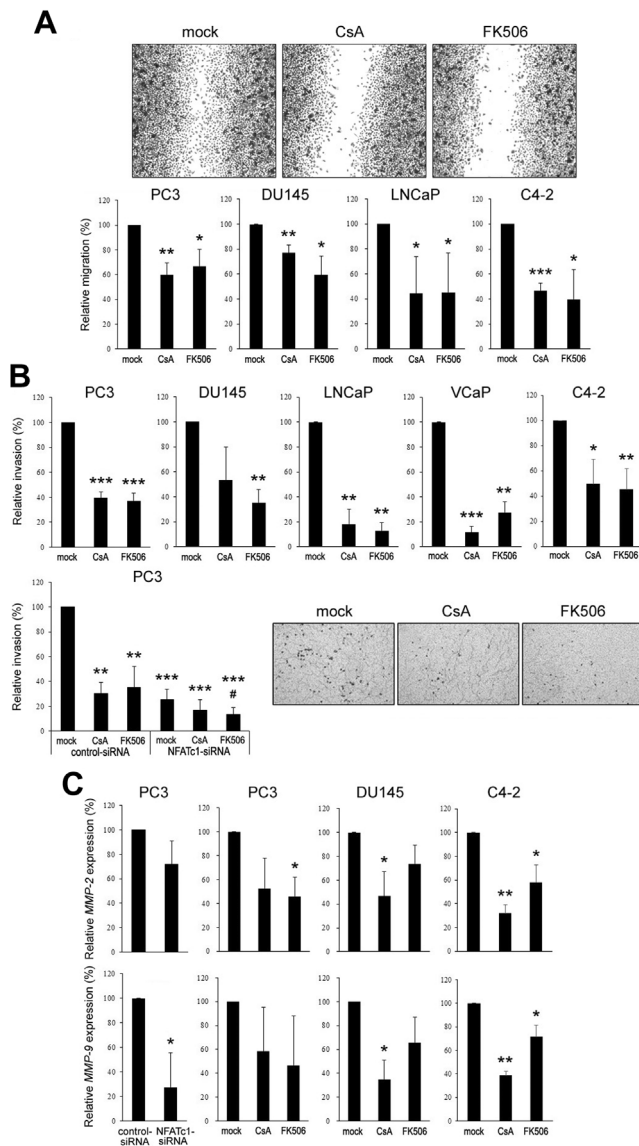
Fig. 4. Effects of NFAT inactivation on cell migration and invasion. **A**: Wound healing assay in PC3/DU145/LNCaP/C4–2 cells. The cells grown to confluence were gently scratched and the wound area was measured after 24 hr culture with ethanol (mock), CsA (1 μM), or FK506 (1 μM). The migration determined by the rate of cells filling the wound area is presented relative to that of mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *$P < 0.05$ (vs. mock treatment in each cell line). **$P < 0.01$ (vs. mock treatment in each cell line). **$P < 0.001$ (vs. mock treatment). **B**: Transwell invasion assay in original PC3/DU145/LNCaP/VCaP/C4–2 cells and PC3 expressing control-siRNA or NFATc1-siRNA. The cells were cultured in the Matrigel-coated transwell chamber for 24 hr in the presence of ethanol (mock), CsA (1 μM), or FK506 (1 μM). The number of invaded cells present in the lower chamber was counted under a light microscope (10×objective in five random fields). Cell invasion is presented relative to that of mock treatment in each cell line. Each value represents the mean (+SD) from three independent experiments. *$P < 0.05$ (vs. mock treatment). **$P < 0.01$ (vs. mock treatment in

each cell line). ***$P < 0.01$ (vs. mock treatment in each cell line). #$P < 0.05$ (vs. mock treatment in NFATc1-siRNA cells). **C**: Quantitative RT-PCR of *MMP-2* and *MMP-9* in prostate cancer cells. PC3 cells expressing control-siRNA or NFATc1-siRNA and original PC3/DU145/LNCaP/VCaP/C4–2 cells were treated with ethanol (mock), CsA (1 μM), or FK506 (1 μM) for 24 hr and subjected to real-time RT-PCR. Expression of *MMP-2* or *MMP-9* was normalized to that of *GAPDH*. Transcription amount is presented relative to that of mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *$P < 0.05$ (vs. mock treatment in each cell line). **$P < 0.01$ (vs. mock treatment in each cell line).

100 mm^3, we commenced daily injections of CsA or FK506 (Fig. 5A). The inoculated tumors in mice treated with CsA or FK506 were significantly smaller than those in the control mice at 6–18 days of treatment. CsA and FK506 also prevented the growth of tumors exceeding 1,000 mm^3. Immunohistochemical staining in the harvested specimens revealed decreases in cell proliferation as the percentage of Ki-67-positive cells (Fig. 5B) and NFATc1 expression (Fig. 5C) in CsA/FK506-treated tumors. These in vivo data further suggest that CsA and FK506 strongly inhibit the progression of prostate cancer and help determine the clinical relevance of our in vitro findings.

DISCUSSION

A wide range of biological functions of NFATs in the immune system have been recognized. Similarly, increasing evidence suggests their involvement in the progression of hematological malignancies as well as solid tumors. Although the NFAT pathway has been implicated in its growth using in vitro models [14–17], specific NFAT isoforms have not been studied in prostate cancer. In the present study, we demonstrate our data indicating that NFATc1 inactivation via NFATc1-siRNA as well as treatment with NFAT inhibitors, CsA and FK506, results in prostate tumor regression.

Unique functions of each NFAT isoform in not only the immune response but also cell differentiation, neoplastic transformation, and tumor outgrowth have been indicated [5–13,24]. It is also likely that individual NFAT isoforms regulate downstream targets and subsequent tumor progression or regression in a cell type-dependent manner. Several studies have demonstrated that NFATc1 induces tumorigenesis and tumor progression [7,10–12]. Recently, we also showed that NFATc1 inactivation resulted in suppression of bladder carcinogenesis [25] and cancer growth [26]. On the other hand, a few studies suggested the promoting roles of NFATc2 in the progression of malignancies, including pancreas [27], breast [28], and colon [29]
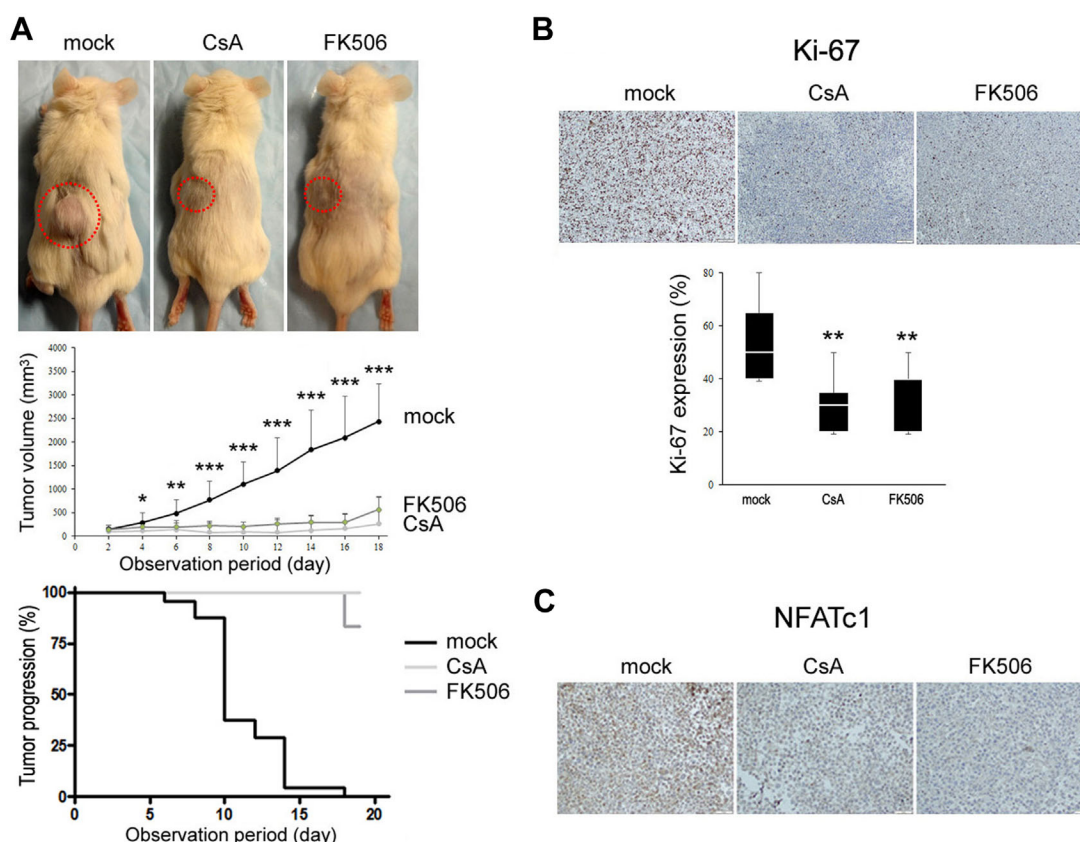
Fig. 5. Effects of CsA and FK506 on tumor growth in a mouse xenograft model for prostate cancer. **A**: CsA (30 mg/kg/day), FK506 (3 mg/kg/day), or vehicle control was injected intraperitoneally in PC3-bearing NOD-SICD mice. Tumor size was monitored every other days. *$P < 0.05$ (mock vs. CsA). **$P < 0.01$ (mock vs. CsA)/0.05 (mock vs. FK506). ***$P < 0.001$ (mock vs. CsA or FK506). Kaplan–Meier curves and log-rank test according to the endpoint set as tumor volume exceeding 1,000 mm^3 are also shown. **B**: Immunohistochemical staining of Ki-67 in harvested xenograft tumors. Mean values ($\pm$ SD) of the percentage of Ki-67-positive tumor cells are shown. **$P < 0.01$ (vs. mock treatment). **C**: Immunohistochemical staining of NFATc1 in harvested xenograft tumors. Diffuse (mock) or focal (CsA/FK506) NFATc1 signals are seen.

cancers, while it was clearly shown to function as a tumor suppressor in a sarcoma model [10]. We here demonstrated that NFATc1 knockdown resulted in retardation of prostate cancer cell growth, and no additional inhibition by NFAT inhibitors was seen in NFATc1-silencing cells. In contrast, in NFATc1-siRNA cells, NFAT inhibitors, especially FK506, further inhibited their invasion. These findings suggest NFATc1- dominant regulation of prostate cancer cell proliferation and the involvement of the pathways other than NFATc1 (i.e., other NFAT isoforms, non-NFAT) in its invasive properties. Indeed, anti-proliferative effects of CsA via the non-NFAT pathway have been reported in colon cancer models [30].

CsA and FK506 have been shown to inhibit androgen-induced growth of LNCaP cells, presumably via blocking or attenuating androgen binding to AR, AR nuclear translocation, and AR transactivation [16,17]. The proliferation of AR-negative PC3 and DU145 was also inhibited by CsA, but not by

FK506 [16]. Partially inconsistent with these findings, in our assays under androgen-depleted conditions, CsA and FK506 similarly retarded cell growth of AR-negative (PC3, DU145), AR-positive/androgen-sensitive (LNCaP, VCaP), and AR-positive/castration-resistant (C4–2) lines, implying the unnecessity of androgen-mediated AR signals in NFAT-mediated regulation of prostate cancer cell proliferation. Also, inconsistent with previous observation in LNCaP [16], CsA and FK506 induced apoptosis in PC3, DU145, and C4–2. In addition, we demonstrated, for the first time, inhibition of cell migration and invasion by CsA and FK506 in prostate cancer lines. However, the AR pathway may involve this, because more significant effects were seen in androgen-sensitive cell lines than in AR-negative or AR-positive/castration-resistant cells.

The blood concentrations of CsA (e.g., 1 µM) or FK506 (e.g., 25 nM) are often strictly adjusted in transplant recipients, because their clinical use at high

doses can cause serious side effects [31]. Thus, FK506 can achieve similar immunosuppression with up to 50-fold lower doses than CsA [32]. In our cell line assays, the same concentration (1 μM) of CsA and FK506 showed similar inhibitory effects, whereas, in our xenograft model, 30 mg/kg/day CsA and 3 mg/kg/day FK506 achieved similar retardation of tumor growth. Therefore, CsA may be more potent in inducing prostate cancer regression than FK506 at their pharmacological concentrations. Indeed, a pharmacological dose (25 nM) of FK506 did not significantly inhibit NFAT expression in prostate cancer cells and their viability (data not shown). As aforementioned, in prostate cancer lines, CsA was shown to more strongly inhibit cell proliferation, compared with FK506 at the same concentrations up to 10 μM [16]. Again, in that study [16], CsA inhibited the growth of both androgen-sensitive and AR-negative prostate cancer lines, while FK506 inhibited only AR-dependent growth. These findings suggest some differences in detailed mechanism of action for prostate tumor regression, as observed in NFAT-mediated immune response, as well as safety, between CsA and FK506. To prevent severe adverse drug reactions potentially seen with CsA or FK506 that ubiquitously inactivates the NFAT pathway, more specific inhibitors of NFAT isoforms (e.g., NFATc1) may need to be developed.

Elevated expression of NFATc1 has been detected immunohistochemically in several types of malignancies, other than prostate cancer. In these studies, subcellular localization of NFATc1 appeared to be cancer type-dependent: predominantly nuclear in liver cancer [9], both nuclear and cytoplasmic in pancreatic [7] and lung [8] cancers, and predominantly cytoplasmic in subcutaneous T-cell lymphoma [33]. Recently, we also detected both nuclear and cytoplasmic signals of NFATc1 in non-neoplastic urothelium and urothelial tumor specimens [25,26]. Our current immunohistochemistry for NFATc1 in prostate TMAs exclusively stained the cytoplasms of benign and malignant epithelial cells. More importantly, NFATc1 was significantly higher in prostatic carcinoma than in benign or HGPIN and in HGPIN than in benign, suggesting its role as a promoter in prostate carcinogenesis. In accordance with these staining results, our RT-PCR showed higher NFATc1 expression in prostate cancer cell lines than in benign cells. Furthermore, NFATc1 levels strongly correlated with tumor grade, but not tumor stage, as well as patient outcomes. In particular, multivariate analysis revealed that NFATc1 levels independently predicted tumor recurrence. These findings thus support other data suggesting that NFATc1 promotes prostate cancer progression.

NFATc2 expression determined by a semi-quantitative RT-PCR was much weaker in androgen-sensitive prostate cancer cell lines than in benign cells, suggesting its role as a tumor suppressor. Nonetheless, NFATc2 was overexpressed in castration-resistant C4–2 cells derived from androgen-sensitive LNCaP and was also considerably expressed in AR-negative PC3 and DU145. It also seemed that NFATc1 expression in C4–2 was stronger than that in LNCaP. Therefore, NFATc2 and NFATc1 may contribute to the emergence of castration resistance that remains a major clinical problem in the treatment of prostate cancer. All the cell lines examined, except VCaP, were found to similarly express NFATc3, NFATc4, and NFAT5, respectively. Further assessments of each NFAT isoform function are necessary for determining biological significance of NFAT signaling in prostate cancer progression.

CONCLUSIONS

NFATc1 likely plays an important role in prostate cancer progression. Our findings may also offer a potential therapeutic approach for prostate cancer via targeting the NFAT pathways, especially NFATc1 signals. CsA (and FK506) may thus be able to be applied to the treatment of advanced prostate cancer, including castration-resistant tumor. However, mechanistic details underlying the suppressive effects of NFAT inhibitors may need to be further characterized.

ACKNOWLEDGMENTS

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